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THE BOTANICAL REVIEW

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LIFE-FORMS AND PHYTOCLIMATE

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> C'est dans la beauté absolue des formes, c'est dans l'harmonie et dans le contraste qui naissent de leur assemblage, que consiste ce que l'on nomme le caractère de la nature dans telle ou telle région.
>
> A. von Humboldt, 1807.

Probably every science has had periods in its development when some phase was in fashion and courted by the scientifically sophisticated. In botany, emphasis has at one time or another been placed on taxonomy, geography, ecology, physiology, vascular anatomy, embryology, cytology, and genetics. In the same way, ecology has experienced vogues in the study of physiological ecology, growth-form adaptation, succession, quadrat analysis of the community, atmometry, hydrogen-ion concentration of the soil, osmotic concentration of cell sap, pollen analysis, etc. Such peaks of activity often result from the initial efforts of some leader or the development of suitable instruments and techniques. Always there is an over-emphasis during such phases of activity, while the usefulness of the technique or point of view is finding its place in the whole field of study; and there often follows a period of unpopularity and neglect of the field. The latter phase does not result from the fact that the subject has been exhausted, nor even that the cream has been skimmed from it, but partly at least as a reaction to over-emphasis and false or too broad claims for the results. Such has been the fate of the study of life-forms and physiognomy of vegetation.

A BRIEF HISTORICAL SKETCH OF LIFE-FORM STUDY

Although man has always been aware of certain major life-form differences among plants (grass, herb, tree, shrub, undershrub—Theophrastus), his early interests were in the uses of plants and, as the science of botany developed, in their description and classification. His first interest in plant communities was floristic, and it was not until Humboldt (39) that the concept of growth-form was formalized. Humboldt attempted a grouping of vegetational types on a physiognomic basis, and there was some but not much effort on his part to express relations between the environment and such life-form groups as the cactus-form and the banana-form[1]. After several decades Kerner (45) established a series of purely morphological forms as distinct from the systematic position of plants in the various genera and families. Grisebach (34) built his system on Humboldt's ideas and emphasized the dependence of life-forms upon climate. Drude (26), who has been followed in more or less detail by many later students of the problem, emphasized the role played by the particular species in the vegetation and its life history, especially duration, protection and propagation, under the prevailing conditions of the habitat. A quarter of a century later (27) he attempted to classify the ecologically uniform members of systematic groups upon a combined morphological-taxonomic basis.

Warming (66) considered the adaptation of plants to the unfavorable seasons of the climate, to the conditions prevailing during the growing season, and to the conditions of the soil. In his classification he emphasized: *a*) the duration of the vegetative shoot; *b*) the length and direction of shoot development; *c*) the position and structure of the renewal buds; *d*) the size of the plant; *e*) the adaptations of the assimilation organs with respect to water loss; *f*) the competitive and social capacities of the plant. Schimper (61) reemphasized the physiological-adaptational point of view. Pound and Clements (53) developed a system that owed much to Drude and Schimper, and was modified to fit the conditions in our Central States. With some further modifications, this system is restated by Clements (23) in his monograph on plant succession and indicators.

The most popular and widely used system of all, that of

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[1] In 1807 Humboldt named the following 15 life-form groups whose physiognomy forms an important study in the description of landscapes: *a*) the banana form (*Musa, Heliconia, Strelitzia*); *b*) the palm form; *c*) arborescent ferns; *d*) the form of *Arum, Pothos* and *Dracontium*; *e*) the conifer form (*Taxus, Pinus*); *f*) all the sharp-leaf forms; *g*) the tamarisk form (*Mimosa, Gleditschia, Porlieria*); *h*) the mallow form (*Sterculia, Hibiscus, Ochroma, Cavanillesia*); *i*) lianas (*Vitis, Paullinia*), *j*) orchid form (*Epidendrum, Serapias*); *k*) the cactus form; *l*) the casuarina form and *Equisetum*; *m*) the grass form; *n*) the mosses; *o*) lichens.

Raunkiaer (54, 56), emphasizes the position of the perennating or renewal buds. Although at first glance Raunkiaer's system looks very different from earlier ones, particularly because of the single theme of the major classes, there is a strong dependence upon materials used by earlier students in the sub-divisions of the classes. Raunkiaer's system will be considered in some detail in later sections, for it is the only system which has been widely used.

Because any system will have some weaknesses, and because points of view differ, there continue to be proposed various organizations of life-form classes. Brockmann-Jerosch and Rübel (17) and Rübel (59, 60) may be cited as examples embodying earlier work together with distinctive ideas. Gams (31) developed the synusial concept of community structure[2], and went so far, as did Vahl (65) before him, as to base the classification and nomenclature of plant communities upon life-forms. Hayek (37), in his general plant geography, presents a system with 100 life-form groups. The most recent thorough-going study of the life-form problem is that of Du Rietz (28) who has abandoned all suggestions of adaptation or epharmony. Lippmaa (46, 47) follows Vahl and Gams in emphasis upon the synusiae in vegetation study.

Such a brief historical survey as this leaves out of consideration many studies that have been made and, without giving details for any of the systems, it suggests that there has been varying emphasis upon morphological types, upon growth- or life-forms in which there is a presumed direct relationship between environment and form, and upon taxonomic entities.

After a strong interest in the life-form problem around the turn of the century when ecology was developing as a recognized science, there has been little sustained interest in life-forms, especially in America, and no system other than Raunkiaer's has been used widely. The reasons for this neglect, other than the complexity of many of the systems, are that American ecologists have been little interested in detailed studies of community composition and structure, and have been more interested in succession and studies of the factoral composition of the environment. There has also been some adverse reaction to life-form study because of physiological investigations which have shown that a plant's functioning is not always what it would seem to be on a basis of structure—that structure and function are not uniformly one—and that adaptation may not reveal itself on the surface. The first duty of the ecologist, however, is to the plant and to the plant community; and the life-form, whatever its adaptational significance, is an important part of vegetational description ranking next, perhaps, to the floristic composition of the community.

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[2] A synusia is a minor community, such as a layer or bark community, within a complex community, dominated by a single life-form or by closely related life-forms.

THE CONCEPT OF LIFE-FORM

What we have been referring to as life-form (growth-form or vegetative-form) has nothing whatever to do with reproductive structures which constitute the principal basis of classification into species, genera, families, etc. Life-form refers rather to the vegetative form of the plant body which is assumed by many ecologists to be a result of morphological adjustments to the environment. Those organisms which show the same general morphological features (woody lianas, stem succulents, annuals, taprooted perennials with a basal rosette of leaves and the renewal bud at the soil surface, tall broad-leaf deciduous trees, etc.) belong to the same life-form whatever their systematic position in the plant families. It is inherent in the so-called "biological" concept of life-form that there is a fundamental harmony or analogy between the members of such structural groups and the environment in which they prevail. This relation is denied by others.

In the century and a half since Humboldt developed the concept of life-form, botany has become a much deeper and broader science, and many earlier interpretations (and some recent ones) are not tenable. What is meant by morphological adjustments to the environment are not those comparatively minor differences which exist among members of the same species grown in different habitats (ecads), such as sun and shade forms, but long-time adjustments that have an evolutionary basis and have become fixed in the heredity of the kind. No longer do any biologists except those of Lamarckian persuasion believe that the environment "calls forth" adaptational responses. The harmony or fit between the organism and the environment is the result of natural selection of a long series of mostly minute mutational changes.

Despite many individual exceptions and despite the fact that adaptations to a particular environment may consist of many types, there is still a widespread correlation between certain life-form types and certain environmental types, and on this basis there persists a belief in the significance of life-forms for environmental adaptation. A few examples will show what is meant. Under certain extreme environmental conditions, such as great drought or a long cold winter with a very short growing season, plants of the tree form cannot exist, i.e., in deserts, steppes, and arctic and alpine tundra. Furthermore, tree stature is progressively reduced from the most favorable environments to the continental and altitudinal tree limits. In American deserts the succulence of the cacti is one adaptation that is matched by the euphorbias of South African deserts. In deserts, wherever they occur and by whatever floristic groups they are populated, the development of microphylly, aphylly, and switch-like shoots are common forms, as is the ephemeral habit of development in annuals and perennials. The grass form prevails in steppes and is common in tundra. Temperate climates with strongly contrasting winters and summers but without drought seasons are characterized by broad-leaved deciduous trees with abundant hemicryptophytes[3] and geophytes[3] in the ground layer beneath them. Where the winter is colder and the growing season shorter, evergreen needle-leaved trees predominate. In the hot humid tropics are developed the largest trees with broad, oval, shining leaves and with feathery, pinnate, evergreen leaves. Here in numerous families also occurs the conspicuous development of the woody liana form and of large epiphytes among ferns and seed plants.

But the life-form problem is not a simple one. No environmental type is characterized by a single life form except in the most narrow sense of the biotope and the synusia. That is, all the larger environmental types, the climates and the major associated soil types, have plant communities composed of several life forms. Furthermore, no life-form class is associated solely with a single environmental type. Since there is no life-form exclusiveness, since the harmony between structure and environment is not complete, the problem is a complex one and has been dealt with in many ways.

Many ecologists are content with a consideration of or at least emphasis on the dominant layer of the vegetation. Thus a forest is studied as if it were composed of trees alone, or a prairie only of grasses. At the other extreme all the synusiae of the phytocoenosis[4] are examined in detail. Many ecologists indicate the life-form of each component species of a community. Thus, in the simpler systems such as that of Raunkiaer, life-form groups can be given a statistical treatment and the enumerations for different examples of a community type can be compared, and those of different kinds of communities can be contrasted.

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[3] See pp. 7-11 for definitions.

RAUKIAER'S LIFE-FORM SYSTEM

Any life-form system has the following broad philosophical basis: *a*) plants have different ecological amplitudes, that is, they are more or less limited in their capacity to endure different environmental conditions; *b*) there is often a correlation between morphology and adaptation; and *c*) a plant in its successful existence represents what may be called an automatic physiological integration of all of the factors of its environment.

Raunkiaer employed three guiding rules in the selection of life-form characteristics for the recognition and classification of the relationships between plant life and climate: *a*) the character must be structural and essential; it must represent an important morphological adaptation; *b*) the character must be sufficiently obvious that one can easily see in nature to which life-form a plant belongs; *c*) all the life-forms employed must be of such a nature that they constitute a homogeneous system; they must represent a single point of view or aspect of plants and thus enable a comparative statistical treatment of the vegetation of different regions.

With these requirements in mind, Raunkiaer delimited life-forms by the kind and degree of protection afforded the growing points of the perennating buds which are responsible for renewal after an unfavorable season. Except for certain tropical climates that are constantly warm and humid, they all show a certain amount of seasonal rhythm with alternating periods that are favorable or unfavorable for growth. In comparing two climates, the difference between them in their favorable season may be relatively insignificant, whereas the differences in their unfavorable season may be considerable and of great importance. Unfavorable seasons may be due to cold or drought or both, they may be short or long, there may be one or two each year, but it is probable that those structural differences among plants that enable them to survive unfavorable seasons are a good index to plant climates. Upon these facts, then, Raunkiaer developed his life-form classification which depends primarily upon the degree of protection afforded the perennating bud.

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[4] A phytocoenosis is the whole vegetation of a station or place, a community in the usual broad sense. It consists of the many synusiae or layers of plants which occur at the same station.

The system consists of the following five principal classes arranged according to increased protection of the buds: phanerophytes, chamaephytes, hemicryptophytes, cryptophytes, and therophytes.

CLASS I. Phanerophytes (Ph) have their bud-bearing shoots in the air and are with few exceptions woody trees and shrubs. Since these buds are elevated and exposed to the atmosphere during the unfavorable season, and since the severity of conditions increases with height above the ground, the phanerophytes are logically subdivided into height classes: tall, medium and low trees, and shrubs. These subdivisions have been formalized with more or less arbitrary boundaries as the megaphanerophytes (Mg), over 30 meters tall; the mesophanerophytes (Ms), between eight and 30 meters tall; the microphanerophytes (Mc), between two and eight meters high; and the nanophanerophytes (N) which are under two meters and over 25 cm. tall. The relationship between height and environment is seen in a number of correlations. Most species show a certain amount of plasticity with respect to stature. Some are facultative shrubs; that is, they are trees under more favorable conditions and shrubs under less favorable environments. For example, *Quercus stellata* develops as a mesophanerophyte in Tennessee and Arkansas and is gradually reduced in stature toward the west as the climate becomes drier until vast stands may be only a few feet tall in the Oak Shinnery. Similar reductions take place in may tree species toward artic and alpine tree lines. There are, of course, hereditary limits for the stature of plants so that mature heights are fairly well known for the most favorable sites for various tree species. In a general way the tallest trees occur in the most favorable climates, in the warm, humid tropical rainforests, in the more favorably located temperate deciduous forests and needle-leaved conifer forests; and for any of these types stature is reduced on less favorable soils and under worse climates.

In addition to height in relation to the exposure and adaptations of vegetative structures, Raunkiaer also considers whether the perennating bud is without bud scales and whether the transpiring organs are deciduous or evergreen. He therefore provides for 12 subclasses on a basis of these characters as follows: *a*) evergreen megaphanerophytes without bud scales; *b*) evergreen megaphanerophytes with bud scales; and *c*) deciduous megaphanerophytes with bud scales. The fourth possibility, deciduous megaphanerophytes without bud scales, occurs very rarely and is not allotted a separate subclass. Each of the other height classes has the same three subdivisions. Three additional subclasses which do not fit into this scheme are the herbaceous and epiphytic phanerophytes of the tropics and stem succulents of deserts. No special provision is made for woody lianas which are classified according to the height class to which they attain. Lianas are the phanerophytia scandentia of Braun-Blanquet (15).

Under some conditions all these phanerophytes are lumped together and expressed in one percentage. Commonly, however, the height classes (without further distinction as to evergreen, etc.) are kept separate in statistical treatments, except that the two tallest classes are often lumped together under the symbol MM, and separate percentages are given for epiphytes and stem succulents. Braun-Blanquet (15) recommends the usage of only six main groups of phanerophytes: *a*) nanophanerophytes; *b*) macrophanerophytes (Raunkiaer's Mc, Ms, and Mg classes); *c*) stem succulents (including certain members of the Cactaceae, Euphorbiaceae, Compositae, Bombacaceae, Sterculiaceae); *d*) herbaceous phanerophytes (*Musa, Piper, Impatiens, Begonia,* Euphorbiaceae, etc.); *e*) woody lianas, which are set off from the first two groups; *f*) vascular epiphytes. Each of these groups may be subdivided on a basis of many characters according to their prevalence in a particular community and their adaptational significance. For example, epiphytes can be classified as semiparasites, those with stem tubers, with aerial roots, or with clustered humus- and water-collecting leaves. And nanophanerophytes and macrophanerophytes can be subdivided according to leaf size, leaf form, leaf duration, etc.

Not only are the phanerophytes as a group most exposed to such unfavorable conditions as may occur, but their subdivisions can be arranged in a fairly well graded sequence according to their prevalence in various climates so that we find the evergreen megaphanerophytes without bud scales in the tropical rainforests, and with them are associated concentrations of herbaceous phanerophytes, and epiphytes and lianas which may make up 10% to 15% of the total flora (44). And at the bottom of the scale are the deciduous nanophanerophytes with bud scales which are not uncommon dominants in deserts and shrub steppes, and with which are associated the succulents.

CLASS II. Chamaephytes (Ch) have their perennating buds definitely above the soil surface but lower than about 25 cm. so that in the unfavorable season they often are protected by fallen leaves and snow or by the dense growth of the plant itself. Raunkiaer (54) recognized four main subdivisions according to shoot behavior: *a*) suffrutescent or semi-shrubby forms; *b*) those that are passively decumbent; *c*) those that are actively creeping; *d*) cushion plants. This class of life-forms has come in for more criticism than any other. Raunkiaer makes a strong case for its climatic significance in the positive correlation of this class' percentage with increasingly high latitude and altitude. But the class is also 50-100% higher than the normal spectrum in such widely different vegetation types as subtropical evergreen forest, Mediterranean olive-type woodlands, and certain steppes. Allan (6), for example, points out that certain New Zealand floras have similar percentages of chamaephytes as Iceland and the climates are wholly different. Percentages of total chamaephytes can have little significance when remote regions are to be compared, and one must consider whether they are herbaceous or woody, evergreen or deciduous, etc. In addition to such subclasses as have been mentioned, some authors have separate groups for the hard grasses of the tussock form, succulent-leaved perennials (*Sedum, Sempervivum, Mesembryanthemum*), creeping herbs (*Thymus, Veronica, Cerastium,* certain grasses), and non-vascular forms such as *Sphagnum,* carpet mosses (*Thuidium, Hypnum, Hylocomium*), and lichens of the fruticose form (*Cladonia, Cetraria*). Most statistical comparisons, however, leave out of consideration the non-vascular species. In this class the chamaephytes that provide the greatest protection of the buds are undoubtedly the very compact cushion or polster-form plants. These are fairly common in certain dry steppes (*Anabasis aretioides* in Algeria), in alpine tundras (*Silene, Androsace, Draba*), and in paramos.

CLASS III. Hemicryptophytes (H) have their renewal buds at the surface of the ground and are thus one step further in the scale of protection than the chamaephytes. They are very numerous in humid temperate regions and often constitute half or more of the total species of an area, particularly in deciduous forests and grasslands. They are also common in tundra except under the most extreme conditions. Raunkiaer distinguished three principal subtypes which are usually grouped together in statistical studies: *a*) the non-rosette or protohemicryptophytes; *b*) the subrosette, and *c*) the rosette types. The first type is without basal leaves, the second type has both basal and stem leaves, and the last type has all or nearly all of its leaves in a compact basal rosette. Each group can be subdivided according to whether stolons are produced. Additional subtypes that have been suggested include herbaceous lianas that die back to the soil surface (Leguminosae, Convolvulaceae), many soft grasses and sedges, and non-vascular plants (bryophytes, lichens, algae). The hemicryptophytes are a large and complex life-form group, and the lumping together of all of them in statistical treatments probably obscures all but the most general relationships to environment.

CLASS IV. Cryptophytes (Cr), the "hidden plants," have their buds beneath the substratum, in the soil, in water, or in the soil under the water. They are, consequently, much more protected than plants whose perennating buds are on the surface or elevated into the air. Raunkiaer recognized three principal subdivisions: geophytes, hydrophytes, and helophytes. The geophytes (G) have tuberous subterranean organs filled with stored food which enables them to make a quick vegetative development with the return of favorable conditions. These perennating structures are commonly bulbs (*Allium*), corms (*Arisaema*), rhizomes (*Podophyllum*), stem tubers (*Solanum*), or root tubers (*Orchis*). Another group forms a transition with the hemicryptophytes (*Cirsium*) in that the buds are at the base of the stem, but below the surface of the ground. Geophytes are common in the Mediterranean type of climate, in some steppes, and in the vernal flora under deciduous temperate forest where they develop rapidly before the full leaf canopy is displayed. The hydrophytes (Hy) include such free floating forms as *Lemna* and *Utricularia* and those which may be rooted but not emergent during the unfavorable season (*Potamogeton*). The helophytes (He) are marsh plants in which the perennating bud is rooted in the soil beneath the water. These are mostly emergent plants. In statistical treatments the two classes are lumped together and given the symbol (HH).

CLASS V. Therophytes (Th) are annual plants, that is, their perennating bud is that of the embryo contained in the seed, as no other embryonic part lives through the unfavorable season. This class represents the acme of protection to the renewal tissues. Annuals are particularly abundant in desert climates and in weed communities which develop where native vegetation is disturbed. They are also fairly common in steppes and other climates which offer short growing seasons except that they are conspicuously poorly represented in tundra. They are also very rare in tropical rainforests and infrequent in primeval temperate deciduous forests. A number of the other life-form groups are simulated by annuals during their vegetative phases. They include forms similar to protohemicryptophytes, herbaceous lianas, and even hydrophytes (some species of *Potamogeton, Najas*). Raunkiaer includes winter annuals with summer annuals on the assumption that the dry summer is more unfavorable to them than the winter period.

RAUNKIAER'S NORMAL SPECTRUM

We have seen that the lack of exclusiveness of the life-forms, that is, the facts that more than one life-form is associated with a climatic type and that no single life-form is limited to a climatic type, leads naturally to statistical comparisons which show the percentage composition of the life-form classes in different areas and different types of vegetation. Broad comparisons are often made on a basis of the five major classes of life-forms; more detailed studies employ various subclasses. From the beginning Raunkiaer intended statistical use of the life-form classes, and in order to provide a sort of yard-stick or base, he developed a so-called "normal spectrum" which represents the world-wide distribution of species among the life-form classes. The normal spectrum is the result of a sampling procedure in which 1000 entities were selected from the world flora in such a manner as to represent a random sample. The results for the major classes are: Ph 46%, Ch 9%, H 26%, Cr 6%, and Th 13%. In more detail, the results from Raunkiaer's calculations (55) are shown in Table 1.

TABLE 1. RAUNKIAER'S NORMAL SPECTRUM, 1916 CALCULATION (55)

Symbol		Classes	Class percentages		
(S)		Stem succulents	2		
(E)		Epiphytes	3		
(MM)		Mega- and mesophanerophytes	8		
(Mc)		Microphanerophytes	18		
(N)		Nanophanerophytes	15		
	(Ph)	Total phanerophytes		46	
(Ch)		Chamaephytes	9	9	
		Total epigeal life-forms			55
(H)		Hemicryptophytes	26	26	
(G)		Geophytes	4		
(HH)		Hydro- and helophytes	2		
	(Cr)	Total cryptophytes		6	
		Total hypogeal life-forms			32
(Th)		Therophytes	13	13	13
			100	100	100

On a basis of the same 1000 species, Raunkiaer also computed normal spectra for the principal systematic groups of the seed-bearing plants, Table 2. Whether the normal spectrum represents accurately the whole world flora does not really matter; its usefulness lies in its being a standard for comparison.

TABLE 2. THE 1000 SPECIES OF THE NORMAL SPECTRUM TREATED TAXONOMICALLY. DATA FROM RAUNKIAER (56)

Taxonomic groups	Ph	Ch	H	Cr	Th
Gymnospermae	100.0	0.0	0.0	0.0	0.0
Angiospermae	....	...	...	...	...
Dicotyledonae	....	...	...	...	...
Choripetalae	59.8	9.7	15.1	1.5	13.8
Gamopetalae	34.9	11.6	36.4	3.0	14.0
Monocotyledonae	27.0	5.0	36.9	21.3	9.6
All species together	46.0	9.0	26.0	6.0	13.0

LIFE-FORM SPECTRA AND PHYTOCLIMATES

Raunkiaer thought that there are four major phytoclimates: *a*) the phanerophytic climate of the warm humid tropics; *b*) the

TABLE 3. LIFE-FORM SPECTRA FOR THE FOUR MAJOR CLIMATIC TYPES ACCORDING TO RAUNKIAER (56)

Locality	Number of species	Life-form classes				
		Ph	Ch	H	Cr	Th
The phanerophytic climate:						
St. Thomas and St. Jan	904	*61*	12	9	4	14
Seychelles	258	*61*	6	12	5	16
The therophytic climate:						
Death Valley, Calif.	294	26	7	18	7	*42*
Argentario, Italy	866	12	6	29	11	*42*
The hemicryptophytic climate:						
Altamaha, Georgia	717	23	4	*55*	10	8
Denmark	1084	7	3	*50*	22	18
The chamaephytic climate:						
Spitzbergen	110	1	*22*	60	15	2
St. Laurence Is., Alaska	126	0	*23*	61	15	1

hemicryptophytic climate of the midlatitudes, including both the needle-leaved and deciduous forests as well as the moister steppes; *c*) the therophytic climate of tropical and subtropical deserts; and *d*) the chamaephytic climate of high latitudes and altitudes. These

TABLE 4. SELECTED RAUNKIAERIAN LIFE-FORM SPECTRA FOR CERTAIN MAJOR CLIMATES

Vegetation type, climate and locality	Data source	No. spp.	Life-form classes				
			Ph	Ch	H	Cr	Th
Rainforest, Queensland	Cromer and Pryor (24)	141	*96*	2	..	2	..
Subtropical evergreen, Matheran, India	Bharucha and Ferreira (9)	361	*63*	*17*	2	5	10
Desert, Transcaspian lowlands	Paulsen (51)	768	11	7	27	*14*	*41*
Mediterranean olive forest, Crete	Turrill (64)	1571	9	*13*	27	10	*38*
Steppe, Akron, Colorado	Paulsen (52)	79	..	*19*	*58*	8	15
Temperate deciduous broadleaf forest, Connecticut	Ennis (29)	1453	15	2	*49*	*22*	12
Tundra, Spitzbergen	Raunkiaer (56)	110	1	*22*	*60*	*15*	2
Normal spectrum	Raunkiaer (56)	1000	46	9	26	6	13

major climates are epitomized by the spectra presented by Raunkiaer, Table 3. There are, of course, subtypes within the major climates and intergradations among them. I have gathered a further selection of "types" in Table 4.

TABLE 5. CORRELATIONS OF RAUNKIAERIAN SPECTRA WITH ALTITUDE

Locality and source	Altitude	No. of species	Life-form classes							
			MM	M	N	Ch	H	G	HH	Th
Clova, Scotland Raunkiaer (56), Table 33	1000 m. +	11	—	—	—	27	64	9	—	—
	900–1000 m.	44	—	—	2	25	52	14	—	7
	800– 900 m.	72	—	—	3	22	60	11	—	4
	700– 800 m.	170	—	1	5	11	67	11	1	4
	600– 700 m.	206	2	2	4	15	62	9	2	5
	500– 600 m.	182	3	3	4	13	63	8	2	4
	400– 500 m.	193	3	3	4	11	66	8	1	4
	300– 400 m.	211	3	3	4	10	65	8	2	5
	300 m. –	304	3	2	4	7	59	7	5	13
Poschiavo, Alps, Raunkiaer (56), Table 23	2850 m. +	51	—	—	—	35	61	2	—	2
	2550–2850 m.	199	—	—	—	25	67	4	—	4
	2250–2550 m.	348	—	1	3	18	64	7	1	6
	1900–2250 m.	492	—	1	3	13	68	8	1	6
	1550–1900 m.	487	1	3	4	11	62	10	1	8
	1200–1550 m.	449	3	3	4	7	60	9	1	14
	850–1200 m.	604	2	3	5	5	55	9	2	19
	850 m. –	447	3	4	3	5	55	8	1	21
Alps, nival zone, Raunkiaer (56), Table 24	3600 m. +	6	—	—	—	67	33	—	—	—
	3300–3600 m.	12	—	—	—	58	42	—	—	—
	3150–3300 m.	19	—	—	—	58	42	—	—	—
	3000–3150 m.	42	—	—	—	53	45	—	—	2
	2850–3000 m.	117	—	—	—	33	62	2	—	3
	2700–2850 m.	148	—	—	—	33	61	3	—	3
	2550–2700 m.	229	—	—	1	28	65	2	—	4
	2400–2550 m.	323	—	—	1	24	67	4	—	4
Mt. Cook, New Zealand, Allan (6)	2000 m. +	22	—	—	—	64	36	—	—	—
	1500–2000 m.	64		....8....		48	44	—	—	—
	1000–1500 m.	219		....15....		33	48	...3...		1
	500–1000 m.	275		....18....		31	42	...6...		3
Clavering Island, Greenland, Gelting (33)	1250 m.	3	—	—	—	67	33	—	—	—
	1200–1250 m.	9	—	—	—	33	37	—	—	—
	1125–1200 m.	21	—	—	—	34	52	14	—	—
	1100–1125 m.	36	—	—	—	41	41	15	3	—
	1000–1100 m.	46	—	—	—	37	50	11	2	—
	900–1000 m.	63	—	—	—	34	50	14	2	—
	800– 900 m.	76	—	—	—	34	50	13	3	—
	700– 800 m.	81	—	—	—	34	50	14	2	—
	600– 700 m.	89	—	—	—	35	49	13	2	1
	500– 600 m.	105	—	—	—	34	49	14	2	1
	400– 500 m.	114	—	—	—	33	50	13	2	1
	300– 400 m.	120	—	—	—	33	48	15	2	2
	200– 300 m.	123	—	—	—	34	47	15	2	2
	100– 200 m.	131	—	—	—	32	47	15	4	2
	0– 100 m.	145	—	—	—	31	49	15	4	2

The correlations of life-form spectra with altitude, Table 5, reveal one significant trend, a steady increase of chamaephytes with altitude although the data come from mountainous areas as widely separated as Greenland, the Alps, and New Zealand. At Poschiavo the percentage of chamaephytes that equals the normal spectrum (9%) occurs at about 1700 m. altitude; below that elevation the percentage is less, and above it, it rises to about four times the normal at 2850 m. Elsewhere in the Alps, where elevations are higher, the percentages increase to over seven times that of the normal spectrum, or 67 per cent at 3600 m. When these results are compared with Clova, Scotland, it is seen that the maximum altitude at Clova of 1000 m.+ has 27 per cent chamaephytes, whereas in the less oceanic Alps at a lower latitude such a percentage is not reached below 2500-2700 m. altitude. The effect of latitude is strongly shown by the Greenland data where, for Clavering Island, the 30% chamaephytic line is at sea-

TABLE 6. CORRELATIONS OF RAUNKIAERIAN SPECTRA WITH LATITUDE

Locality and source	Latitude	No. of species	Life-form classes					
			Ph	Ch	H	G	HH	Th
Novaya Zemlya, Raunkiaer (56), Tables 7 and 8	76–77° N.	4	—	50	50	—	—	—
	75–76° N.	16	—	31	69	—	—	—
	74–75° N.	49	2	25	63	10	—	—
	73–74° N.	128	2	22	62	11	3	1
	72–73° N.	130	2	20	60	12	3	3
	71–72° N.	130	3	20	64	11	2	—
	70–71° N.	141	3	20	62	12	3	—
(Vaygoch)	69–70° N.	154	3	18	63	12	4	—
	(Yugor)	119	3	17	63	11	4	2
East Greenland, Raunkiaer (56), Table 15	76,31–77,36° N.	28	—	36	57	7	—	—
	73,30–75,00° N.	102	—	25	61	10	2	2
	70,00–73,30° N.	159	1	26	55	12	4	2
	69,25–70,00° N.	96	2	32	55	6	2	2
	66,20–69,25° N.	76	3	32	58	5	1	1
	65,30–66,20° N.	169	1	21	60	7	7	4
West Greenland, Raunkiaer (56), Table 16	Lockwood Isl.	4	—	75	25	—	—	—
	N. of Melville Bay	93	1	29	57	12	1	—
	72,00–74,30° N.	123	2	31	56	9	2	1
	69–71° N.	214	2	19	58	12	6	3
	64–67° N.	239	2	19	57	10	8	5
	60–62° N.	254	2	16	56	11	8	7
New Zealand, endemic species, Allan (6)	42–46° S.	177	23	12	63	.. 1 ..		2
	34–42° S.	166	62	2	19	.. 13 ..		4
	34–38° S.	78	65	2	15	.. 14 ..		4
	34–36° S.	30	53	3	13	.. 20 ..		10

level, and at 1250 m. altitude the percentage of chamaephytes is equal to 3600 m. in the Alps. The data from the southern hemisphere are in line with the others, despite the totally different flora and important differences in the types of chamaephytes that are involved. Hemicryptophytes are always abundant in an area where chamaephytes are abundant, but they show no such consistent trend. These, and numerous other data not cited, seem to substantiate Raunkiaer's claims for the significance of chamaephytes as indicators of varying degrees of extremeness in boreal, arctic, and alpine climates (Table 6), for entirely similar trends occur with increasing latitude as with increasing altitude.

It is of considerable interest to examine a series of spectra for Raunkiaer's hemicryptophytic climate. Nearly all of the areas represented in Table 7 are from deciduous forest regions, although there are in some cases admixtures of climax grassland and in others of climax needle-leaved forest. In all cases they represent the total flora of the named areas and, as a consequence, all the combinations of local plant communities as occur under secondary succession, different soils, and different microclimates. They can, then, be said to reflect to a certain degree the general climate, that is, if there is any real relationship between climate and life-form statistics. Although there are evident differences, all these spectra of far-flung places are of the same general pattern, and all these areas belong to the warm-temperate rainy climates of Köppen with hot to warm summers (mostly types Cfa, Cfb, and Dfa). In the American series there are data for several States from the Gulf coast to New England and from the Atlantic coast to, but not including, the BS climates to the west. But the spectra do not appear to reflect sensitively the comparatively minor climatic transitions that occur. We have here only a general correlation between Raunkiaer's hemicryptophytic phytoclimate and Köppen's C and D climates which are based on physical data. Perhaps that is enough to expect from such an approach; yet it may be possible to employ life-form data so that they will reveal more information.

Ewer (30) divided the State of Illinois into four areas: north, central, mid-south, and south. There is a progressive increase in phanerophytes (15.2, 12.7, 16.2, 18.1) with decreasing latitude (Table 8) except for the central area. This departure in the central area would seem to be explained by the larger proportion

TABLE 7. COMPARISONS OF RAUNKIAERIAN SPECTRA FOR TEMPERATE, BROADLEAF DECIDUOUS FORESTS, ON A BASIS OF FAIRLY LARGE AREAS

Localities and sources	No. of species	Life-form classes								
		Mg	Ms	Mc	N	Ch	H	G	HH	Th
North America										
South–north series:										
Mississippi Ennis (29)	1724	3.5	5.4	4.6	4.2	3.1	49.4	12.4	3.8	12.8
Alabama Ennis (29)	2012	2.9	4.8	4.6	4.7	3.1	47.8	12.8	4.3	14.4
Altamaha, Georgia Raunkiaer (56)	717	(5.0)		7.0	11.0	4.0	55.0	4.0	6.0	8.0
N. C.–Tenn., Smoky Mts. Cain (19)	1142	2.5	6.4	6.1	4.5	1.7	52.1	15.1	0.0	11.5
Bull Run, Virginia Allard (7)	980	1.8	6.4	5.6	4.4	1.4	51.7	9.1	2.2	17.0
New York, local flora Taylor (62)	1907	0.5	4.0	7.2	3.5	5.3	33.3	20.2	11.7	13.0
Connecticut Ennis (29)	1622	1.4	4.0	5.6	3.5	2.0	49.4	12.6	7.7	13.5
Cape Breton Ennis (29)	637	1.4	2.9	3.7	6.1	1.8	51.3	15.3	10.3	6.7
East–west series:										
Indiana McDonald (48)	2109	(14.3)				1.9	49.0	(18.0)		16.7
Illinois Ewer (30)	1734	1.4	5.0	6.0	3.6	1.3	49.7	13.1	5.5	14.1
Iowa Ennis (29)	1320	2.2	3.7	4.8	4.1	1.0	48.6	15.3	5.6	14.2
Europe										
Serbia Turrill (64)	2734	0.0	1.7	2.8	3.4	11.3	46.2	9.1	4.7	20.7
Stuttgart Raunkiaer (56)	862	(3.0)		3.0	3.0	3.0	54.0	10.0	7.0	17.0
Poschiavo under 850 m. Raunkiaer (56)	447	(3.0)		4.0	3.0	5.0	55.0	8.0	1.0	21.0
Central Switzerland Dansereau (25)	....	(10.0)				5.0	50.0	(15.0)		20.0
Paris region, Vexin Allorge (8)	....	(8.0)				6.5	51.5	(25.0)		9.0
Denmark Raunkiaer (56)	1084	0.0	1.0	3.0	3.0	3.0	50.0	11.0	11.0	18.0
Asia										
Japanese-Hondo Horikawa and Sato (38)	3601	0.4	7.8	11.2	8.2	2.0	47.4	8.0	3.7	10.0

Table 8. Comparison of Raunkiaerian Spectra for Four Latitudinal Areas of Illinois. Ewer (30)

Areas of Illinois	Life-form classes								
	Mg	Ms	Mc	N	Ch	H	G	HH	Th
Northern area	1.3	3.7	6.0	4.2	1.4	50.0	14.0	5.0	14.1
Central area	1.3	4.2	4.7	2.4	0.8	51.9	13.5	4.9	16.1
Mid-south area	2.2	6.2	5.0	2.8	0.7	51.0	12.6	3.5	16.0
Southern area	2.5	7.4	6.2	2.0	1.0	49.3	12.0	4.5	14.9

of prairie in that area. Conversely, the hemicryptophytes reach their largest percentage in the central area. The strong increase in phanerophytes in the southern area is largely attributable to elements of the southern flora of the Mississippi embayment which reach their northern limits there (*Taxodium distichum, Quercus phellos, Nyssa aquatica, Aesculus pavia,* etc.). When lianas alone are considered, their percentage in the northern area is 1.2 and is exactly doubled in the southern area. It can be noted that the Köppen climatic classification recognizes a transition between types in southern Illinois. Ackerman (1) has published a revised map

Table 9. Raunkiaerian Spectra for Merriam's Life-zones in Washington. Jones (42, 43)

Life-zones	Life-form classes				
	Ph	Ch	H	Cr	Th
The Olympic Peninsula					
Arctic-alpine zone	0	21	69	9	0
Hudsonian zone	9	10	67	13	1
Canadian zone	12	7	49	31	1
Transition	15	3	43	25	14
Mount Rainier					
Arctic-alpine zone 6,000–14,408 ft.	0	19	69	10	0
Hudsonian zone 4,500–6,000 ft. Chamaecyparis climax	9	14	61	13	1
Canadian zone 3,000–4,500 ft. Tsuga-Abies-Pinus climax ...	11	8	47	31	3
Transition zone 2,000–3,000 ft. Tsuga-Thuja climax, and Pseudotsuga subclimax	16	4	43	26	16

of the Köppen climates of North America which shows that all of Illinois is Dfa climate except for the southern tip which is Cfa. This is based on the position of the January 0° C. isotherm.

One approach to a greater refinement in the usage of life-form spectra as indicators of similarities and differences between different areas is found in the studies of Jones in the State of Washington. For the flora of the Olympic Peninsula (42) separate spectra are provided for the various life-zones of Merriam. With increasing altitude the life-zones are Transition, Canadian, Hudsonian, and Arctic-alpine. In the order of these zones there is a regular increase of chamaephytes and hemicryptophytes, being much stronger in the former, and a regular decrease of phanerophytes, cryptophytes, and therophytes, with increasing altitude (Table 9). In a detailed study of Mount Rainier, Washington, the climaxes are identified with the Merriam life-zones (43). The five prin-

TABLE 10. COMPARISONS OF RAUNKIAERIAN SPECTRA FOR CERTAIN FOREST TYPES OF THE TEMPERATE, BROADLEAF, DECIDUOUS FOREST

Vegetation type, location, and source of data	No. of species	Life-form classes				
		Ph	Ch	H	Cr	Th
Oak-hickory climax, Piedmont of North Carolina, Oosting (50) ..	89	59.6	0.0	36.0	4.5	0.0
Mixed mesophytic cove hardwoods, Smoky Mts., Tenn., Cain (19) ..	113	36.3	4.4	30.1	25.8	3.4
Mixed mesophytic climax, Cincinnati area, Withrow (68)	127	33.6	3.9	34.4	23.4	3.9
Upland forest, Cincinnati area, Withrow (68)	94	49.8	4.2	23.4	15.9	6.4
Mountain-oak woods, Long Island, Cain (18)	92	34.8	10.9	32.6	20.6	1.1
Sugar maple woods, Laurentian area, Dansereau (25)	346	17.0	10.0	56.0	15.0	2.0
Beech woods, South Cevennes, Braun-Blanquet (13)	...	2.0	4.0	51.5	40.5	2.0
Aspen association, Michigan, Gates (32)	310	22.9	3.9	47.1	16.1	10.3
Aspen association, Alberta, Moss (49)	170	25.8	1.8	48.2	17.1	7.0
Beech woods on chalk, England, Clapham (Tansley and Adamson) (20)	...	23.0	7.0	56.0	15.0	0.0
Scottish beechwood, adult, Watt (67)	37	24.5	16.0	46.0	13.5	0.0
Scottish heathy beechwoods, Watt (67)	90	13.5	18.0	53.0	13.0	2.0

cipal classes of life-forms there show trends similar to those for the Olympic Peninsula (Table 9).

Another refinement is in comparisons of spectra for individual plant communities, rather than for the total floras of extensive areas. In Table 10 is a series of spectra for particular associations of the temperate deciduous forest formation. Here we see somewhat more sensitive comparisons than existed for the more general spectra of the preceding tables for State floras. For example, two very similar mixed mesophytic forests of the Great Smoky Mountains and of the Cincinnati area have very similar life-form spectra. In contrast, a related climax type, the sugar maple forest of the Laurentian area, shows the effects of latitude through the reduction of phanerophytes from around 35 to 17%, and an increase of chamaephytes from about four to 10% and of hemicryptophytes from 30 to 56%. These changes seem much more significant and true to the climatic differences than the general spectra discussed earlier. In another case the very similar aspen associations of Michigan and Alberta have strikingly similar life-form spectra. Thus it would seem that extensive series of such data for comparable communities, and also with information on the subclasses, might provide quite close correlations with climates, at least within the C and D climates of Köppen.

Turning to the dry climates (BW, desert; BS, steppe), we find a more complicated situation. In Table 11 I have assembled all the spectra for dry climates that I have been able to locate. At first there appeared to be a great variety, but when arranged according to the Köppen climates that are involved, some interesting and probably significant correlations appear. The two monsoon spectra are not very similar. This may be because the Tuticorim spectrum is essentially of a strand flora; yet they are of a type with the principal difference being that Tuticorim has more annuals and fewer woody plants than Madras. The Madras spectrum is, incidentally, very similar to that for Aden. Actually there are too few data to make much speculation worthwhile for the humid tropics.

The spectra for hot desert climates seem to fall into two groups: the North African deserts have relatively few phanerophytes in comparison with the other examples, while in the Australian desert they are especially numerous. In all the deserts annuals are abun-

TABLE 11. SOME LIFE-FORM SPECTRA FOR DRY CLIMATES, COMPARED WITH KÖPPEN TYPES

Locality and source	Köppen class	No. of spp.	Life-form classes							
			S	MM	N	Ch	H	G	HH	Th
Tropical monsoon climate with long dry summer:										
Madras, India Bharucha and Ferreira (9)	Am	689	0	(34)		23	10	5	0	15
Tuticorim, India Børgesen (12)	Am	35	0	3	20	28	8	3	0	37
Hot desert climate:										
Death Valley, California Raunkiaer (56)	BWh	294	3	2	21	7	18	2	5	42
Salton Sink, California Paulsen (52)	BWh	81	0	0	33	6	(14)		0	47
El Golea, Central Sahara Raunkiaer (56)	BWh	169	0	0	9	13	15	5	2	56
Ghardaia, North Africa Raunkiaer (56)	BWh	300	.3	0	3	16	20	3	0	58
Libyan desert, N. Africa Raunkiaer (56)	BWh	194	0	3	9	21	20	4	1	42
Oudjda desert, N. Africa Braun-Blanquet (16)	BWh	49	0	0	0	4	17	6	0	73
Oudjda semi-desert Braun-Blanquet (16)	BWh	32	0	0	0	59	14	0	0	27
Canary Islands lowlands Børgesen (11)	BWh	176	0	2	17	19	10	4	0	47
Aden Raunkiaer (56)	BWh	176	0	7	26	27	19	3	0	17
Ooldea, Australia Adamson and Osborn (5)	BWh	188	4	19	23	14	4	1	0	35
Cool desert climate:										
Transcaspian lowlands Paulsen (51)	BWk	768	0	0	11	7	27	9	5	41
Hot steppe climate:										
Tucson, Arizona Paulsen (52)	BS	266	0	0	18	11	(24)		0	47
Whitehill, South Africa Adamson (4)	BSh	428	1	1	8	42	2	18	0	23
Timbuctu, Africa Hagerup (35)	BSh	138	1	11	12	36	9	3	3	25
Tripoli, North Africa Raunkiaer (56)	BSh	369	0	.3	6	13	19	9	2	51
Cyrenaica, North Africa Raunkiaer (56)	BSh	375	0	1	7	14	19	8	0	50
Madeira Islands, lowlands Raunkiaer (56)	BSh	213	0	1	14	7	24	0	3	51
Cool steppe climate:										
Akron. Colorado Paulsen (52)	BSk	379	0	0	0	19	58	8	0	15
Toole, Utah Paulsen (52)	BSk	116	0	0	2	23	46	3	0	14

TABLE 11.—*Continued*

Locality and source	Köppen class	No. of spp.	Life-form classes							
			S	MM	N	Ch	H	G	HH	Th
Cool steppe climate—*Continued:*										
Danube steppe, S.E. Europe										
Bojko (10)	BSk	?	0	0	7	5	55	10	0	23
Yekaternoslaw, Near East										
Paulsen (51)	BSk	1046	0	0	5	3	55	8	5	24
Pamir mountain steppe										
Paulsen (51)	BSk	514	0	0	1	12	63	5	5	14
"Mediterranean climate" warm temperate with hot dry summer:										
Argentario, Italy										
Raunkiaer (56)	Csa	866	0	6	6	6	29	0	2	42
Southern France										
Braun-Blanquet (14)	Csa	?	0	0	7	13	29	8	0	43
Crete										
Turrill (64)	Csa	1571	0	4	5	13	27	10	2	38
Samos, Greece										
Raunkiaer (56)	Csa	400	0	5	4	13	32	11	2	33
"Mediterranean climate" warm temperate with warm dry summer:										
Table Mountain, S. Africa										
Adamson (2)	Csb	93	0	4	37	14	18	19	0	7

dant, sometimes composing more than half the flora, except for the dubious case of Aden. Hemicryptophytes are fairly constant, but that heterogeneous class, the chamaephytes, are quite variable. If detailed data were available it would likely show that the mere fact of the perennating bud being above the soil but less than 25 cm. high is of much less adaptational significance than are some other features which would be revealed by a consideration of subclasses. Deserts are well known for the fact that plants have found many different types of adaptation to the conditions of life. The single example of a cool desert, the Transcaspian lowlands, shows the coolness, perhaps, in the increased proportion of hemicryptophytes and cryptophytes.

The spectra for grassland climates show some conspicuous variations. In general the drier steppes have much smaller percentages of hemicryptophytes than do the steppes with more rainfall—compare, for example, Timbuctu and Akron, or Cyrenaica and the Danube steppes. Annuals play a variable role which may be difficult to resolve because over-grazing, which is so prevalent in grasslands, tends to increase the percentage of therophytes through the introduction and spread of weedy grasses and forbs of this life-form. The chamaephytes are again variable and the remarks that were made concerning them for deserts would hold true also for steppes.

The correlations between the spectra for the Mediterranean "olive climate", with warm to cool moist winters and hot dry summers (the most favorable seasons are spring and fall), are strikingly good. The climate of southwest Africa is of the same general type except that the summer is less hot. The spectrum, however, is so different from those of the Mediterranean region that one wonders whether the life-form classes employed are adequate for the comparison of such distinctive floras. On the other hand, there is a possibility that the physical climatology is partially in error and this South African region should not be classified as a "Mediterranean" type. It would be interesting to have life-form data from southern California, coastal Chile, and southwestern Australia where the Cs climate also occurs.

The significant deviation of the Csb Table Mountain spectrum (South Africa) from Csa Mediterranean spectra—notably the excess of nanophanerophytes and geophytes at the expense of hemicryptophytes and therophytes—may have a floristic basis in the excess of certain phylogenetic stocks hereditarily disposed toward one life-form or another. If this is true, the phenomenon strikes at the heart of the concept of life-form harmony with climate. One can seek other explanations such as a relative excess of nanophanerophytes and geophytes due to repeated burning of the vegetation (2, 3). The vegetation of Table Mountain used for the spectra is not of the climatic climax type and may, as a consequence, not truly represent the Csb climate. This, however, is more or less true of the basis of many other available spectra, and the question needs further study.

FREQUENCY SPECTRA

In the paragraphs on the spectra for the temperate deciduous forest region some data were given that seemed to indicate that climatic differences may be more sensitively reflected by spectra for associations than by spectra for the total flora of large areas. Another statistical refinement was introduced by Raunkiaer in his community studies. In all the previous spectra we have cited, each species is counted as one in the statistics, no species having more weight in the results than another, irrespective of the role played by a species in the vegetation of the area. In a large area where many different communities occur it would be very difficult if not impossible to assign all the species various values, but within a single community certain sampling methods permit each species of the community to be given a value that is some measure of its role in the community. These data may be actual abundance, but are more often frequencies which, under certain sampling procedures, may approach true densities. The most significant data would be some measure of the relative dominance of the species in the community. In any case, life-form spectra that are based upon the role of the species in the community are of more value than those in which the rarest and most incidental members of the community carry as much weight in the spectrum as do the species that are dominant in the superior layer and the various other synusiae.

TABLE 12. FREQUENCY SPECTRA FOR COVE HARDWOOD HERBACEOUS COMMUNITIES

	No. of species	Life-form classes				
		Ph	Ch	H	Cr	Th
Great Smoky Mts., total flora ..	1142	19.5	1.7	52.1	15.1	11.5
Cove hardwoods, total flora	113	63.3	4.4	30.1	25.8	3.4
Cove hardwoods, field layer,						
Vernal aspect community	72	...	7.0	47.2	40.3	5.5
Aestival aspect community ...	75	...	6.6	61.3	29.3	2.6
	Frequency points					
Cove hardwoods, field layer,						
Vernal aspect	1309	...	11.0	43.3	41.9	3.8
Aestival aspect	1258	...	14.2	58.6	23.9	3.3

One illustration of this technique is given in Table 12 by data from the Great Smoky Mountains (19). The first spectrum is for the total flora of the area, and the second is for the cove hardwoods, a primeval, mixed—mesophytic climax forest association. The third and fourth spectra are for the spring and summer herbaceous layer communities under the cove hardwoods. All these spectra are based solely on species lists in which one species carries as much weight as another. The last two spectra are based on frequency points. The vernal flora was sampled by 100 separate one sq. m. quadrats (ten each in ten different stands of the forest): each species was given as many frequency points as quadrats in which it was found. The summer community was sampled in the same manner except that the quadrats were of six sq. m. area. The larger sample area was used because the summer vegetation is of much greater stature than the spring flora[5].

The use of frequency data in the computation of life-form spectra, in comparison with spectra based on species occurrence alone, shows the relatively more important role played by chamaephytes whose percentages increase from 7.0 and 6.6 to 11.0 and 14.2.

TABLE 13. A COMPARISON OF LIFE-FORM AND FREQUENCY SPECTRA. DATA FROM RAUNKIAER (57)

		Ch	H	G	Th
Community A:	species spectrum: 18 spp.	11.1	66.7	22.7	....
	frequency spectrum: 504 pts. ...	17.5	42.0	40.5	....
Community B:	species spectrum: 42 spp.	4.8	83.3	9.5	2.4
	frequency spectrum: 1848 pts. ..	4.1	83.8	8.5	3.2
Community C:	species spectrum: 16 spp.	12.5	68.4	19.3	....
	frequency spectrum: 456 pts. ...	12.3	75.0	12.5	....
Community D:	species spectrum: 31 spp.	9.7	80.6	9.7	....
	frequency spectrum: 848 pts. ...	15.5	56.5	27.8	....

Cryptophytes (in this case all geophytes) show up somewhat more in the vernal flora (40.3 to 41.9%) and somewhat less in the aestival flora (29.3 to 23.9%). In comparison with the total Smoky Mountains flora, the cove hardwoods herbaceous communities are seen to be comparatively high in geophytes in the spring aspect and in hemicryptophytes in the summer aspect. The whole procedure, however, does not bring out any very great difference between spectra computed on a basis of species as such and those computed from total frequency points attained by all species of each life form. There are very good reasons for this. In these communities we are dealing with relative life-form dominance of several species (3) rather than with the exclusive dominance of one or two species. That is, instead of one or two species completely controlling these herbaceous communities through their superior adaptations and competitive ability, there is a grand mixture of many species of each life-form type that is abundant.

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[5] Incidentally, the soundness of this procedure is shown by the correspondence in total number of frequency points for the two seasonal communities.

The data in Table 13 from Raunkiaer (57) show that a community that is rich in species often will have a frequency spectrum that is greatly different from the species spectrum. But the most important cause of a strong difference between the two types of spectra resides in the dominance: when a small number of species of the same life-form are very numerous they will have a low percentage in the life-form spectrum and a much higher percentage in the frequency spectrum.

It seems quite clear that for the characterization of individual stands of the various communities of an area the simple species spectra are sometimes of little or no value, but that frequency spectra, which take into consideration the role of each species in the community, are excellent for the life-form characterization of the communities. Life-form spectra based on the total flora of a sufficiently large area seem to be useful in the indication of the general or prevailing phytoclimate. Within such an area, however, the effects of local environments (microclimates and edaphic conditions) are best revealed when the spectra are modified by the use of quantitative data on the roles of various species in the respective local communities.

CONCLUDING STATEMENT

Two particularly significant points arise in this connection. In the first place it is to be stressed that a biological significance is attached to life-forms by Raunkiaer; that is, he makes an *a priori* assumption that the life-forms which he employs represent adaptations that are of great importance for the survival of the plants and that, as a consequence, they measure phytoclimate. This is a tacit assumption of all the physiological ecologists. Whether or not these life-forms, or any others for that matter, do reflect sensitively the environmental conditions is sometimes an open question, for Raunkiaer's work and that of his followers have received considerable criticism. It would seem, however, that when the life-forms are selected and handled statistically without *a priori* assumptions of their adaptive value, they are capable of reflecting climatic conditions in terms of both general climates and microclimates. The second point is that with all assumptions of epharmony aside, life-forms are necessary to the inductive study of vegetation. That is to say, an important part of any vegetational study is the straight factual description of the physiognomy of the vegetation and of the life-forms of the component species, especially those that are dominant in the various synusiae and those that are more or less peculiar to or prevalent in the community or climatic type.

Finally certain relationships between plant geography and taxonomy should be pointed out. On a basis of certain recent reviews of the history of plant geography and ecology (28, 36, 58) it becomes clear that pre-Linnaean taxonomy had a large element of life-form description for its basis. With the development of the Linnaean sexual system of classification, vegetative structures were relegated to a very minor role; but in modern times, according to Adamson (4), form has regained some of its former importance, notably in the work of Hutchinson (41) on the families of flowering plants and in the systematics of the genecologists, Clausen, Keck, and Hiesey (21, 22). With respect to life-form and plant geography, the earliest systems were purely inductive and the various life-form classes were merely descriptive of form and established for the purpose of describing vegetation. After Darwin such geographers as Warming, Kerner, and Drude saw in the physiognomy of plants a causal relationship of environment, either through the processes of natural selection or by some supposed direct causal effect. This trend probably reached its highest development in the Raunkiaerian life-form system. Finally, there has been a reaction to the teleology that often was involved, and to the too broad claims for relationships, and such students of the life-form problem as Du Rietz (28) have gone back to a purely descriptive basis with no assumptions of adaptation of epharmony between their classes and the environments.

What position should be taken by the ecologist or geographer who is not a specialist in the life-form problem? He should, I believe, consider carefully and in some detail the physiognomy and life-form groups of whatever vegetation he is describing. He can and should point out such cases of simple concomitance as may occur between certain life-forms and certain features of the environments under study. Whether any causality is involved may be left to more refined studies, and to the weight of gradually accumulated circumstantial evidence from numerous similar studies. Such an approach to the life-form problem is not a static nor sterile one, and it avoids all assumptions of unproved relationships. He should not be confined to or bound by any previously developed life-form system. On the other hand, the unnecessary erection of duplicate systems and nomenclature is deplorable. To a very great extent the major classes and subclasses of the Raunkiaerian system are useful for straight description. It is good to work with them because of the already wide usage which the system has enjoyed (and out of more abundant statistics may come information of considerable value), but the classes and the subclasses can be modified as the circumstances warrant, as already done by many investigators such as Braun-Blanquet (15).

A few comments may be useful on what seem to me to be the current difficulties in statistical life-form research and some profitable lines of investigation for the future. A worldwide comparison of bioclimatic zones based on life-form statistics and the major climatic types of the Köppen system would indicate their degree of conformity, and in the absence of coincidence of boundaries might suggest modifications of both the biological and physical concepts of delineation of major natural areas. The great impediment to such investigations, however, is the wide lack of comparable floras, from the point of view of both the taxonomic treatment of the species and the details, provided concerning distribution of the entities. Also, when a similar problem is considered on a smaller geographic scale, there are usually inadequate local floras giving details of distribution. In the United States, for example, there are many State floras, but they are of unequal quality. When artificial geographic units such as States are employed in the construction of life-form spectra for comparative purposes, the possible sensitiveness of the spectra to climatic boundaries is obscured, for nearly every area as large as a State will have two or more climatic types within its boundaries. Furthermore, in most States the published distribution records (by counties) are often inadequate for close comparisons.

Although life-form spectra have been employed mostly for general climatic analysis, there are enough studies available to suggest that they may be even more useful for the analysis of climatic variants and microclimates. Also it seems probable that life-form spectra when based on associations, not in the large sense of the association (cf. Clements) but more or less narrowly conceived, can reflect habitat changes with succession, important edaphic conditions, etc.

The usefulness of life-form spectra for such investigations would seem to depend largely on some means of evaluating the species composing the flora of the communities. The weighting of the species could be accomplished by the use of any quantitative data concerning the species, such as abundance, frequence, or coverage. The use of such data, however, involves one immediately in all the problems of community sampling for phytosociological descriptions. It seems certain that the flora of the whole stand of the community should be used for the construction of the spectra, i.e. the plants of all of the layers taken together. In this case numbers of individuals (often difficult or tedious to obtain) are not comparable from synusia to synusia. A thousand beech trees and a thousand anemones do not have the same significance. If frequency data are to be used, the quadrat size must be adjusted to the synusiae, a larger quadrat for larger life forms and progressively smaller quadrats for the successively inferior layers of the community. Coverage data seem perhaps the most useful, for coverage is more nearly independent of sample-plot size than are density and frequency, provided only that the sample areas are large enough to include the typical flora in its characteristic structural development for the community. For use in life-form spectra, however, the usual unequal coverage classes of the phytosociologists are poor, and equal 5- or 10-per-cent classes probably should be used.

Finally, I believe that life-form spectra for climax or near climax vegetation are the only ones that can reflect sensitively climatic correlations. Spectra for association variants might reflect minor climatic variations. Spectra for successional communities of various sorts might reflect edaphic conditions and give a good measure of the changing environment as succession proceeds. These, then, are some of the lines for future study that should be profitable.

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FUNGICIDES FOR FOREST TREES, SHADE TREES AND FOREST PRODUCTS

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INTRODUCTION

Intensive control measures for forest tree diseases are generally impracticable, and for shade trees are limited in scope by the large size of the plants and the small number ordinarily under a single management. Forest pathology has therefore lagged behind orchard and crop plant pathology in direct control measures, and has contributed little to the number of fungicides usable on living plants or to techniques of application. Helione orange reported successful in medication against a trunk infection (perhaps by antitoxic action rather than as a fungicide) (22), phenyl mercury nitrate found to be a safe antiseptic for asphalt woundpaints (51), acids and aluminum and ferrous sulfates used for soil treatment against damping off of conifers (8) are among the few contributions to useful fungicides from tree disease investigations.

In the field of forest products the situation is quite different. Fungicides, using the term in a broader sense than that of Roberts (33), were in large-scale use on wood before they became established for the protection of plants or products of any other crop. Creosote, zinc and mercury salts, and the chlorinated phenols and phenates first reached the stage of large-scale fungicidal use on wood.

TREE SPRAYING AND DUSTING

In nurseries (8) Bordeaux mixture has been successfully employed against a rust of hemlock, a *Phytophthora* seedling blight and leaf diseases of several conifers and broad-leaved species, but has been only partly successful against others. Spraying and dusting with fungicides in forests would be economically conceivable only in very special cases, and most likely by airplane or some fog device. Spraying of course is practicable for shade trees where large estates, municipalities or commercial tree men have the equipment needed for covering large trees, but most street tree spraying is done mainly because of insects. Fungicides have been recommended, in most cases without convincing experimental evidence, for sycamore anthracnose, horse-chestnut leaf blotch, rust on cedar and leaf diseases of various other hosts. Conifers and broad-leaved trees other than black cherry and American beech are in general little subject to spray injury by standard fungicides. The conifers and particularly black locust among the broad-leaves have very waxy leaves or young shoots, and cannot be covered without wetting agents.

TREE INJECTION

Because of the long life and high individual value of shade trees, they offer a special opportunity for the development of medication. The results obtained with lithium salts against chestnut blight (34, 35) were interesting but not good enough to make practicable the saving of the trees, as may also be said of the unpublished results of C. S. Moses and Berch W. Henry with 8-quinolinol against the Chalara wilt of oaks. Other notable efforts in this direction have been recently reviewed by Stoddard and Dimond (44).

WOUND PROTECTION

No entirely satisfactory wound dressing has been developed. A mix of dry Bordeaux and raw linseed oil has been considerably used (27). Where creosote-tar mixtures have been employed, it has been necessary to protect the cambium of some species at wound margins by tape or shellac. As in the treatment of wounds on animals, strong disinfectants by injuring host tissue may actually increase the infection hazard. An interesting case is that of chestnut blight (unpublished work of J. Franklin Collins) in which it was found that more infections occurred at points where copper nails were driven through the bark than where steel nails were used.

The most efficient use of fungicides to avoid infection of wounds has been against the very important canker stain of the London Plane, proved to be transmitted almost entirely in pruning. Any of a variety of fungicides applied to the tools and ropes used by the pruners ends most of the spread of the disease. The asphalt wound paints commonly used have no fungistatic value against the vascular species of *Endoconidiophora* that causes the disease, but have been made safe against serving as a transmitting medium for the fungus by the addition of 0.2% phenyl mercury nitrate. Of many fungicides tried, this was the only one that was both effective and harmless to the cambium (51).

SEED TREATMENT

This also is a procedure little developed for forest and shade trees. There are no important tree diseases known to be carried on the outside of seeds. The pre-emergence damping-off of broad-leaved species, chiefly caused by *Rhizoctonia* in the soil, offers a possible field for seed treatment. For damping-off of conifers a proprietary mercurial has been reported helpful on seed (32), but most damping-off losses come too long after germination to be affected by seed treatment.

SOIL TREATMENTS

For soil treatment against damping off of conifers (8), formaldehyde has been used to some extent. Ten milliliters per square foot of seedbed is usually sufficient. Ethyl mercury iodide has been successfully employed at a nursery where large amounts of animal manure are applied to the beds, but the safety of the mercurials on most seedbed soils is questionable. Acidifying chemicals have been used. In soil as acid as that of many forest nurseries, loss of exchange bases and injury to soil texture may be expected from such treatments. However, at nurseries with near-neutral soil, and especially where the water supply is somewhat alkaline, sulfuric acid (43) or aluminum sulfate (18) has been successfully employed for many years (8, 19). Frequent watering is required during the emergence period to prevent occasional damage to radicals on soils that are not well buffered. Orthophosphoric acid (8), ferrous sulfate (8) and the less widely tested dithiuram sulfide (32) give similar damping-off control and have the additional advantages that the first supplies phosphate, and the second and third can be used without special watering, since neither has been known to injure conifers. However, these chemicals lack the weed-killing value of sulfuric acid and aluminum sulfate. It is not known how much of the value of the acidifying materials is in fungicidal or fungistatic action, and how much may be due to increasing the resistance of the hosts.

SHORT-TIME PROTECTION OF FOREST PRODUCTS

Wood that has more than 25% or 30% of moisture on the oven-dry basis is subject to damage by three functionally different groups of fungi. The molds, as the term is used in connection with wood, are usually Ascomycetes or Fungi Imperfecti with light-colored hyphae, and are objectionable mainly because of sporophores or spore masses they produce on the surface. Molding of lumber is relatively unimportant, since these spores are removed by planing. The stain fungi are those of the same taxonomic groups that have dark mycelium and thus discolor the wood to the depth reached by the hyphae, causing down-grading for many uses. Both groups are limited to the sapwood, in which they develop quickly, using the food materials stored in it, but having little effect on the cell walls or the major mechanical properties of the wood. The decay fungi, mostly Hymenomycetes, are slower working, but break down the cell walls of sapwood and ultimately of heartwood, destroying its value.

In addition to the organisms that affect wood or fiber, plywood and wood pulp products can be damaged by molds and bacteria that attack the protein or starch-containing glues with which some of them are bonded. Painted wood can be injured in appearance by fungi that discolor the paint. Slime production, mainly by bacteria, interferes with the process of making paper from wood. Against all of these types of difficulty, fungicides have been successfully employed.

Logs (39, 46, 50): On some logging operations it is necessary to store logs for weeks or even months before they can be sawed into lumber. A high degree of protection against surface infection with stain or decay fungi can be obtained if the ends, and spots on the sides where the bark has been knocked off, are sprayed or brushed with a fungicide within 24 hours after cutting. Concentrations recommended for logs are: for ethyl mercuric phosphate, 0.045%; for sodium penta- or tetrachlorophenate, 2.5% if used alone, or 1.4% if with 2.2% borax as adjuvant. The tetrachlorophenate is recommended in the South only for hardwoods. Phenyl mercury oleate in petroleum is also successful.

Green Lumber (29, 39, 46, 50): In the early decades of this century there was a considerable development in the Southern Pine Region of dipping lumber as soon as sawed, primarily to protect it against stain fungi during the seasoning period. Verrall has summarized this development in this journal (46). Solutions of soda (usually a mixture of the carbonate and bicarbonate) in the neighborhood of 5–7% were commonly used at temperatures in the neighborhood of 160° F. until displaced in the main by the newer fungicides described by Verrall. Of these, ethyl mercuric phosphate has replaced the less soluble and more volatile chloride used without heating. It and the chlorophenates, and mixtures of these with each other and with borax that he describes, have proven successful in dilutions that cost for materials about 1¢ per gallon, or 15¢ per thousand board feet of lumber (46, 50). The mercurials favor the development of one of the green forms of *Penicillium* but have the advantage of being less likely to cause dermatitis to the men who handle the freshly-dipped lumber. Fluorides were discarded largely because they favor increase in *Trichoderm viride.* Evidence has been presented that at least part of the apparent stimulation of the tolerant molds by these chemicals is really due to elimination of competing organisms (49). In mixture with sodium chlorophenates the ethyl mercuric phosphate is converted into ethyl mercuric chlorophenate (11); most of the sodium chlorophenate remains unchanged because present in excess. Current treating recommendations are given by Verrall and Scheffer (50).

The impression has prevailed that these water-soluble fungicides would be too readily washed off the lumber if it were exposed to rain in handling. In several trials, washing the treated lumber, begun an hour or more after dipping, had an unexpectedly small effect on the efficiency of the protection (36). This can be explained in the case of the mercurials by the strong adsorption of the fungicide by the wood. In the case of the pentachlorophenate, the pH of the wood of the southern species commonly treated is below 6, and thus in the range in which most of this phenate *in vitro* changes into the relatively insoluble pentachlorophenol. The mixtures containing much borax of course raise the pH of the wood surface, but in neither southern pine nor sweet gum does it appear to be raised sufficiently to maintain the fungicide in the water-soluble phenate form. Heavy rain washing immediately after dipping would be more likely to remove the fungicides.

The adsorption of mercurials by wood results in considerable weakening of the solution during use (47). When the regular .015% solution of ethyl mercuric phosphate has been reduced to a sixth of its original volume by dipping, bio-assay indicates that the residue may have less than one fifth of its original concentration. It appears advisable to bring the dip back to original volume by adding fresh solution as soon as a fourth or a fifth of the solution has been taken up by the lumber. Fortunately there is only slight loss in effectiveness of sodium pentachlorophenate solutions during use.

Increasing the temperature of the dips, and adding wetting agents, have not appreciably increased the effectiveness of the treatments now commonly employed.

The volume of lumber dipped in 1941 was about four billion board feet, about a sixth of the then current cut. With the wartime seller's market, scarcity of labor and at times scarcity of chemicals, the amount of dipping declined sharply.

During the war many mills failed to dry lumber before shipping. This frequently resulted in deterioration in transit, and some cases of continued decay after the wood went into construction. The stain-control dips proved surprisingly effective in preventing both stain and decay infection in this unseasoned material, in some trials keeping green lumber of certain Pacific Coast species in apparently good condition for a year in close-piled stacks in which more than a third of the untreated lumber showed some stage of decay. Borax as a component of dipping mixtures appeared particularly valuable where decay prevention was the principal objective (55).

FUNGICIDES IN PAPER AND FIBERBOARD MANUFACTURE: At paper mills, bacteria and other organisms produce slime that interferes seriously with operations. This is prevented in practice by chlorine alone or with ammonia, or by organic mercurials or chlorophenates, the two latter in much lower concentrations than are used on logs and green lumber. Two chemicals of different types may be used, either alternately or in mixture, in order to forestall the building up of organisms that are resistant to a particular toxicant (2, 3, 10).

It is sometimes necessary to store the "wet-lap" for a time before completing the manufacturing process. Serious losses that formerly occurred from fungi developing during storage are largely avoidable by fungicides applied on the "wet machine" (24). Sodium fluoride, organic mercurials and chlorophenates are all effective for this purpose. Dihydroxy dichlorphenyl methane (42) has also been reported effective.

To increase the durability of insulating board, arsenicals and sodium pentachlorophenate have been employed; with the latter alum is used, insuring a pH at which a high proportion of the phenate will be fixed by the fiber, presumably in the phenol form.

There has been difficulty with the molding of fiberboard containers, which in some cases appears to contribute to the disintegration of the board or the deterioration of the contents. The most effective answer to date appears to be in the use of sodium pentachlorophenate. If added to the "furnish" at the rate of 2% of the weight of the dry fiber when making the paper that is to go into the fiberboard, better protection against delamination is obtained than if it is added only to the glues with which the paper layers are bonded. Addition of 3% to each is probably needed for the best protection under very moist conditions. The same fungicide was found effective for the asphalt-treated paper used to line containers, though somewhat inclined to leach out (16).

CONTAINERS: Fungus growth on wooden boxes received special war-time attention, largely because it obscured labels; in extreme cases decay was also involved. The water-soluble chlorophenates and their mixtures with ethyl mercuric phosphate and/or borax that are useful against stain of lumber are also advisable for boxes, especially if the boxes are made of unseasoned wood. Even if infection has entered the wood, sufficiently concentrated solutions prevent heavy surface growth. Various oil-soluble materials are also effective if the wood is not excessively moist when treated, but these are more expensive and the fire hazard must be considered (36).

Plant bands for use in propagation require treatment to keep fungi from prematurely disintegrating the bands or using the wood of the band as a carbon source for a growth heavy enough to exhaust soil nitrogen. Pentachlorophenol or phenate, and copper naphthenate are regarded as most promising, and are being given commercial trial.

The question has been raised as to whether a fungicide can be incorporated in fiberboard or other container stock or placed inside containers, which would release enough vapor to protect the contents. For this, the use of diphenyl in citrus fruit wrappers supplied a precedent, as also the use of volatile fungicides in optical instruments. Of 46 chemicals tested in vapor form against molds on sapwood, leather, cloth-backed adhesive tape, malt agar, or all of these, paradichlorobenzene appeared the most promising (37). Ethyl mercuric chloride was very effective but is subject to too much question as to its safety for general use in closed spaces or for miscellaneous contents. Ethyl mercuric phosphate was much less effective, probably because of its lower volatility. The studies did not reach the point of practical application.

Prevention of mold in food container stock in general presents a difficult problem because of the possibility of poisoning the food or hurting the flavor. Sodium orthophenylphenate is considered safer than the chlorophenates. Of the chlorophenates, the pentachlor is the safest because of its relatively low vapor pressure. By adding other materials, as borax, the concentration needed can be kept low. Materials at present more expensive that have been effective against molds in tests and might be considered are octadecyl trimethyl ammonium pentachlorophenate and 8-quinolinol. On tomato picking baskets, Plastex, a mixture of coaltar and clay in which one of several fungicides is dispersed (41), is employed in some large operations with reportedly satisfactory results and no damage to contents.

LONG-TIME PROTECTION OF FOREST PRODUCTS

No attempt will be made to cover completely the large literature on wood preservation. For more complete coverage the reader is referred to the standard manual by Hunt and Garratt (23), to the summary from the botanical standpoint by Cartwright and Findlay (6), to the 44 volumes of the annual Proceedings of the American Wood Preserver's Association, and to the Forest Service publications on preservation, a list of which can be obtained on request to the Forest Products Laboratory, Madison 5, Wis. (15). The recent summary by Blew *et al* (4) of American fence-post preservation trials its particularly worth notice.

The attempt to preserve wood led to the first large-scale use of fungicides. Patents on the use of mercuric chloride and of creosote for this purpose were granted in Europe half a century before microorganisms were generally accepted as the cause of either plant or animal disease. An efficient installation for impregnation of timber with mercuric chloride was built in New England 25 years before the discovery of Bordeaux mixture was announced. Originating as an art, the preservation of wood against decay has become largely a chemical engineering project; Federal investigations in it are carried on by the Forest Service, but on some phases pathologists have given assistance. Coal-tar creosote is employed on a scale requiring tankers for its transportation, and large amounts of zinc salts have also been used. One important effect has been to reduce greatly the drain on our forests for poles and ties, and to shift it from the naturally durable woods to the pines, thus averting the rapid exhaustion of the durable species that threatened at the turn of the century. The state of mind in informed circles prior to the general development of preservation was indicated by the fact that the U. S. Bureau of Forestry in 1890 published a bulletin an inch thick on the use of steel ties as a contribution to forest conservation.

The ideal treatment would be one that would protect simultaneously against fungi, insects, fire, and the swelling and shrinking that come with moisture changes. Most of the preservatives used have value against both insects and fungi, and some oil-borne chemicals have more or less water-repellent effect, or can have moisture repellents incorporated with them. Impregnation with wax and more recently with synthetic resins has been publicized as giving multiple protection. A rather high degree of protection against fungi has actually been obtained with absorptions of around 30% by weight of phenolic resin, and in some cases with urea resin, but these make the wood so hard, heavy and expensive as to limit their field of usefulness. The protection they give is probably more physical than chemical.

It is interesting to note that ammonium phosphate and sulfate at the retentions used as fire retardants have also been found to prevent decay in accelerated tests against four fungi, though they permitted or even favored the development of some of the molds (40). This finding is the less surprising, since decay fungi that are able to grow in heartwood are accustomed to a nearly nitrogen-free environment.

Since service tests of wood preservatives require 25 years or more to complete, the need for accelerated tests in this field is particularly acute. Various techniques have been used for testing the fungistatic capacity of proposed preservatives against pure cultures of wood-destroying fungi in agar, wood, wood pulp or sawdust, and for accelerated tests in the open. Methods based on inhibition of spore germination, as used in studying fungicides that are to be sprayed on plants (21), are not adapted to trials of wood preservatives. Simulation of natural weathering of treated wood is necessary and difficult. There is yet no generally accepted quick method for judging probable preservative performance, but encouraging progress is being made (6, 9, 20, 25, 31). Wood-rotting species, and in some cases strains within species, differ in their tolerance to different fungicides. The Division of Forest Pathology, U. S. Department of Agriculture, has about 300 species currently in culture, and the selection from the commoner of these of a sufficiently representative group of test fungi is one of the subjects of study. *Lentinus lepideus* is commonly employed because of its importance in creosoted wood, and *Poria monticola* (*P. microspora*) and *Lenzites trabea* because of their frequency in various forms of timber and tolerance, respectively, to copper and arsenic. *Polyporus tulipiferus* (Madison 517 formerly referred to *Fomes annosus*) and *Poria incrassata* have been much used but are less common in nature. The copper-tolerant *Poria xantha* that frequently damages boats has also been used. In Europe the copper-tolerant *P. vaporaria* and the zinc-tolerant *Coniophora cerebella* have been favored test fungi. Probable persistence of preservatives in service can be inferred to some extent from solubility and vapor-pressure data, and these together with the results of fungistatic tests in agar, or better in wood, are of service in screening the large number of new chemicals and mixtures now available, and reducing them to the few that are worth including in the expensive and long-continued service trials that must be made in different regions before conclusions can be final.

Natural decay resistance of heartwood is a matter of practical interest, even in timber that is pressure-treated with preservatives, since the preservatives do not penetrate the heartwood portions of most species. The resistance of the naturally durable species has been found at least partially due to slightly water-soluble fungistatic materials present in the wood (6, 38, 56). Better knowledge of these natural preservatives may some day serve as a guide to the development of better artificial fungicides.

WATER-BORNE CHEMICALS: Among the chemicals used in wood preservation with water as the carrier, zinc salts have been the most important, while copper and mercury salts, and fluorides, borates, arsenicals, chromates and dinitrophenol or mixtures of these have also been employed. The chromates have been included for their effect in preventing corrosion of metal fasteners by the preservatives, and in the effort to decrease the losses of other salts by leaching, more than for their fungistatic value. Attempts to develop preservatives that can be introduced in soluble form, sometimes by double diffusion, but go into less soluble combinations in the wood, have been made in recent decades. The principal commercial outcomes of these attempts to date have been zinc meta arsenite and chromated zinc, copper, and copper-arsenic (28) preparations. An interesting two-stage treatment given extensive trial on boxes was with sodium pentachlorophenate and copper sulfate, with copper pentachlorophenate an expected product; this was effective against molds but caused skin irritation in subsequent handling of the boxes. No water-soluble materials have thus far been shown equal to creosote for use in situations in which the wood is subjected to leaching, but some of them have the advantage of paintability and lower cost, and have a place, especially for wood that will not be in continued contact with soil or water, particularly if the wood is to be treated while still green.

The safeguarding of glued wood against microorganisms requires protection of some kinds of glue. The synthetic resins with which most of the better plywood and other glued members are bonded are in general little affected by fungi, despite the fact that the phenol, resorcinol and formaldehyde that make up a large part of such resins generally lose their fungicidal value in the polymerization that takes place during gluing. Most of the synthetic resins will probably need the addition of fungicides only if they are much extended by flour or other fungus-susceptible material. However, the protein glues that are used in much plywood are subject to attack by molds, or under very wet conditions by bacteria, and delamination has frequently resulted. Much of the soybean glue in plywood containers for military use during the latter part of the war contained 4% of sodium tetrachlorophenate. This was not entirely satisfactory, but the use of higher concentrations or of the perhaps more effective pentachlorophenates was hampered by their unfavorable effect on the working qualities of the glue. The need in protein glues for high concentrations of fungicides that are effective in agar in hundredths of one percent, is not surprising, since value as a protoplasmic poison is likely to mean ability to combine readily with protein.

OILS AND OIL-BORNE CHEMICALS: Coal-tar creosote is the backbone of the wood-preservative industry. Wood properly impregnated with it normally lasts 25 and often 50 or more years, even in contact with soil or water. The situations to which it is adapted are somewhat limited by its odor, color, tendency to "bleed", and the fact that treated wood can be painted only with aluminum paint. It is a by-product, a mixture of compounds of imperfectly known composition and present in variable proportions. Probably in part because it is a mixture, it is generally effective against all the important wood destroyers, though there is a remarkable species of *Hormodendrum,* a dark mold not perceptibly injurious to the wood itself, which is a regular inhabitant of creosoted wood and is not often found on untreated (7). Laboratory studies in agar have shown the most fungistatic constituents of creosote to be in the low-boiling fractions, but this has not been borne out by tests in wood (12); the persistent quality of its protection in practical experience indicates considerable efficacy also for some of the less volatile constituents. The amount of the relatively inert pitch that creosote may contain, and the degree to which creosote can be extended without loss of efficiency by adding petroleum are questions not yet fully settled. Wood-tar creosotes, water-gas-tar creosote and anthracene oils (carbolineum) have also been used but to a much smaller extent than the coal-tar creosote.

Oil-soluble chemicals that have been used in recent years to a considerable extent include chlorinated phenols, copper and zinc naphthenates, and phenyl-mercury oleate. Of the phenols used, pentachlorophenol is the most lasting because it is less soluble in water and lower in vapor pressure than tetrachlorophenol or 2-chloro-o-phenylphenol. Mixtures of pentachlorophenol with one or both of the other chlorophenols are sometimes used, partly because these mixtures are more easily dissolved in light petroleum solvents. Pentachlorophenol is most readily soluble in petroleums of high aromatic content and is usually supplied as a concentrated solution which can be diluted with any of the ordinary petroleums. During periods of creosote shortage, mixtures of pentachlorophenol and creosote have been used, but with reported difficulty in avoiding sludging at high temperatures unless the water driven out of the wood during treatment is disposed of. Copper naphthenate has also been used in mixture with creosote. Solvents for preservatives used on wood that is to be painted range from mineral spirits to kerosene or even light Diesel oil if absorptions are low; mineral spirits are best if the painting is to be done within a few hours. Copper naphthenate is said in some cases to "bleed" through a single coat of light-colored paint. The phenylmercury oleate is not recommended for wood that is to have contact with soil, and none of these preservatives has been extensively tested for a period long enough to show how it compares with creosote in lasting quality.

PAINT: Paint has less protective value against fungus damage than has sometimes been claimed for it. In some situations exceptionally well maintained paint coats may help very considerably by hindering the pickup by the wood of intermittent moisture, and it may have some fungus-barrier action. On the other hand, if applied to green wood or in any situation in which water gets behind it, it probably favors decay by hindering the evaporation of moisture.

In very moist situations, especially along the coast of the Gulf of Mexico, paints are themselves subject to damage by fungi which can spoil the appearance of houses. Thymol, mercuric chloride, phenylmercuric naphthenate, and chlorophenols or mixtures of these are sometimes added to paint to prevent its discoloration by dark mold fungi. A high percentage of zinc pigment decreases the likelihood of damage (30).

METHODS OF APPLYING PRESERVATIVES: The thorough impregnation that is required for good protection is not easy to obtain. The most certain way to get it is with pressure, aided by heat which decreases the viscosity of the oils used. For preservatives not water-soluble, the wood is usually first seasoned for a time to reduce the moisture content. In the original full-cell process, vacuum was used before introducing the preservative. In a recently developed method for small installations, vacuum is used without either heat or pressure (14). The empty-cell or Rueping technique employs air under pressure at first, then forces the preservative into the wood at still higher pressure, and after some hours releases the pressure so that a considerable part of the preservative is forced out of the wood by the internal air cushion and recovered. This leaves sufficient preservative in the wood for fungus protection and is economical of preservative, and the treated wood is lighter and cleaner. From six to 12 pounds of creosote are ordinarily retained per cubic foot of wood. Treating plant charges for an eight-pound treatment with creosote or chlorophenols have recently been in the neighborhood of $40 per thousand board feet; for water-soluble salts the cost is lower.

Despite the best of treatment there is usually a core of untreated wood. As far as possible boring and fitting should be done before treatment. When this is not feasible, unpenetrated wood exposed in subsequent cutting should be given heavy brush or dip treatment with preservative.

In attempts to avoid the cost of pressure treatment, various efforts are made to get effective treatment without pressure. The method most used is the hot-and-cold bath. The wood is soaked in a hot bath till it is heated through and the resulting expansion of the air in the wood drives much of it out. The wood is then shifted to a cooler bath, or the hot liquid replaced by cold, or the original bath is allowed to cool with the wood still in it, and the contraction of the air in the wood allows the outside air pressure to force in the preservative. The initial heating may be in hot air (23, p. 191) or in boiling water (53), thus avoiding loss of fungicide from vaporization in the hot bath. The hydrostatic pressure obtained by holding the wood at the bottom of a deep vat (5) also helps, but may not increase the depth of penetration as much as it does the amount absorbed. The cold bath should not be too cold, since at low temperatures oils are too viscous for the best penetration.

A device for securing penetration by water-soluble salts in green wood is to apply a mixture of them in the form of a paste which is kept moist for some weeks (23, p. 196). With permeable woods and favorable conditions, fluorides may diffuse deeply; most fungistatic salts do not diffuse so freely. The introduction of preservatives into the sapstream of the living tree or through the ends of freshly-cut logs is discussed by Hunt and Garratt (23, p. 200) and Wilford (54).

Cold bath treatment alone, even when continued for several days, usually gives less penetration. In uninfected wood, penetration across the grain during the first few minutes of such treatment is measured in hundredths of an inch, and hours of soaking may result in surprisingly little additional penetration. However, with a light oil solvent, even at temperatures below the freezing point, long soaking was reported in one set of tests to have resulted in good penetration (52). Movement with the grain is more rapid, depending on the wood structure.

Shallow treatments have little protective value for wood thick enough to develop deep checks, or for any kind of wood that will be in contact with the soil. However, even brush or dip treatments with oil-borne preservatives that can be easily applied on construction jobs, have given indications of value for exterior woodwork that is to be painted and kept above the soil, such as sash, doors, porches, steps, railings, automobile bodies and boats. Joints, which are the places of greatest decay hazard, appear to be particularly benefited by brief treatment (48). In well-replicated experiments in southern Mississippi, joints of nominal 2″ × 4″ sap pine were treated by heavy brushing, or by a short cold soaking, before assembly, so that surfaces in the joints were treated as well as external surfaces. They were then painted and exposed outdoors under conditions that caused early decay in similar assemblies that were given ordinary paint only. All units given a 15-minute soak in 5% pentachlorophenol still appeared good after seven years, and copper naphthenate (0.5% copper) did nearly as well, whereas all untreated units were decayed. It is of course impossible to say how long such protection may last. Pentachlorophenol, and phenylmercury oleate which was also effective but somewhat less so, are subject to slow loss by vaporization, though less so than some of the more toxic components of creosote.

Treatment of wood after it has been put into service has been frequently attempted, but the only large use to which it has led is that in which pentachlorophenol, arsenicals or creosote and sodium fluoride are put into the surface soil around old poles to give them a few additional years of service by retarding external decay (17). It is entirely possible to prevent infection or even stop decay already present, for example, by putting preservatives into holes bored in the wood, or feeding it gradually to wood surfaces by a wick leading from a container; but systems that will maintain a sufficient supply of both preservative and solvent long enough to insure sufficient penetration are not easy to set up in all situations, and have not been intensively studied. The brush or spray treatments often given wood in place may have negative value, since they give a false sense of security and lead to the neglect of such important factors as control of moisture. However, when new untreated lumber is substituted for wood members that have been taken out because of decay, heavy brush treatment is justified of both the new wood and of adjacent wood that is left in place, on the surfaces where they will be in contact. Such treatments used in joints experimentally have hindered the spread of decay fungi out of infected members as well as its entrance into sound members (45).

Large differences are found in wood penetrability (23). Heartwood of most of the important timber species is difficult or impossible to penetrate. Sapwood penetration is much easier in some species than in others. Vessels without tyloses, as in red oak, aid in penetration. In the conifers, permeability tends to be inversely correlated with the proportion of bordered pits in the cell walls that are closed by aspiration of the pit membranes. The effect of previous infection of the wood by bacteria or fungi in the standing tree or in the log, on the penetration of preservatives, needs further study. In laboratory tests wood infected with a staining fungus was more readily penetrated by a staining fungus than was uninfected wood (26). On poles on which shallow decay had developed, spraying with pentachlorophenol in petroleum was reported to have resulted in sufficient penetration to stop the decay (15).

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THE ROLE OF TOXIC SUBSTANCES IN THE INTERACTIONS OF HIGHER PLANTS

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EARLY AND NOT TOO CONVINCING INVESTIGATIONS

That toxic substances may be given out to soil by higher plants and that these substances may affect the growth of the same or other species is a theory which while old is not yet generally accepted. DeCandolle (8) noted that certain species appeared to be specifically inhibitory to the growth of associated species, as, for example, *Euphorbia* versus flax and thistles versus oats. On the basis of these observations deCandolle developed a theory of crop rotation embodying the general idea that each member in a rotation should be a species not inhibited in its growth by toxic substances left from the preceding crop. Liebig (15), while he originally supported the theory of deCandolle, later and as a result of his exhaustive analyses on the depletion of soil minerals by crops, came to the conclusion that not only the amounts but the balances between inorganic materials in the soil are of importance in the growth of plants. On this basis he ultimately discounted the notion that toxic substances may be of importance in regulating crop yields. From the time of Liebig until the present day, plant growth interactions have been almost unanimously interpreted in terms of mineral nutrition effects, or more recently in terms of water competition.

The interest in toxic secretions of plants arose in part from a consideration of so-called soil sickness due to one-crop agriculture. Thus it was frequently observed in early experiments that as a piece of ground was continuously cropped to one plant the yields decreased and that these decreases could not be made up by additional fertilizer. Besides the injurious after-effects or yield-lowering effects of one-crop agriculture, several cases of harmful interaction between plants grown adjacent to each other in a field, or of one crop on a succeeding crop, were also recorded. Thus effects of grass on fruit trees, of walnut trees on other plants, of corn, rye, thistles, turnips, sesame, rutabaga and others, were observed (6, 19, 22, 25). Some species were consistently reported in the early literature easily injured by other plants, as, for example, fruit trees susceptible to injury by grass; grapes, alfalfa and tomato, and others susceptible to injury by various other species.

In the years 1900 to 1910 there was a considerable revival of interest in the possibility of toxic secretions from plants. In 1903 Pickering (20) published the first of a series of works (1, 20, 21) on the injury of apple trees by grass. This effect, which appears to have been a real one, was successively attributed to nutrient competition and to harmful effects of oxygen exclusion from the fruit tree roots by the grass. Each of these possibilities was ruled out on the basis of specific experiments, and the author finally concluded that the pernicious effects of grass were due to a direct poisoning effect on apple roots. The clearest experiments consisted of growing two plants in separate containers, the grass in a tray above the container in which the fruit tree was grown, so that the roots of the two species did not intermingle, but so that the apple roots received the washings of the grass roots. In this way it was noted that the grass inhibited the growth of young apple trees by some means apparently carried from one pot to the other by leachings. This experiment indicates strongly that under some conditions at least some grasses can give out substances which inhibit the growth of apple trees. Whether this effect obtains under field conditions was not clearly resolved by the experiments of Pickering. Russel (26) and Howard (13, 14) have, on the other hand, interpreted the results of Pickering as owing to nitrogen removal by the grass.

Livingston (16), working on the harmful effects of bog soil, showed that waters obtained by leaching a bog soil contain materials which are toxic to the growth of numerous species. In no case were the harmful substances isolated from water obtained by leaching bog soils, but it was noted that the substances were in some cases volatile, and in some cases apparently of colloidal nature.

The best known and perhaps most widely discussed work on toxic substances in soil is that of Schreiner and co-workers in the Department of Agriculture in the early years of the twentieth century (28–34). Schreiner *et al.* found that in certain soils where fertility had been reduced, for example, by cropping to one plant for a series of generations, water extracts could be made which were toxic to the growth of wheat. The toxic principles were isolated and found to be in various cases picolonic acid, salicylaldehyde, vanillin and dihydroxystearic acid. Many other substances were isolated from soil, but only these four were found in amounts sufficient to cause actual toxic activity at the levels in which they occurred, and correlations could be established between infertility of a given soil and presence of one or more of these compounds. Other workers in the field of plant nutrition have pointed out, however, that many causes of soil infertility, in addition to the accumulation of toxic substances, are of importance, and it is undoubtedly true that Schreiner and his colleagues over-emphasized the role of toxic substances in bringing about soil infertility. In an over-all estimate of the work of Schreiner it may be concluded that it did show that toxic substances can accumulate in soils and did identify the chemical nature of these substances. It did not, however, show how the compounds arise from plants presumed to make them, nor did it work out satisfactorily the relative importance of these compounds in the regulation of plant growth in comparison with other factors which may limit yield.

MORE RECENT AND MORE CONVINCING INVESTIGATIONS

With the discrediting of soil toxicity in the general reaction against the work of Schreiner *et al.,* interest in the subject of toxic substances in soil lagged for more than 15 years. In his review of 1937 Loehwing (17) came to the conclusion that the literature up to that time did not provide evidence for any important role of any soluble toxic substance in any normally aerated soil, a conclusion which he shares with Russel (26). In relatively recent years several specific instances of injury to one plant by substances given off by the same or different species have been brought forward. This later work is in general more convincing than the earlier investigations and is in some cases based upon thorough chemical experiments.

One of these newer reports concerns the black walnut tree. This tree has been observed many times to exert detrimental effects on neighboring plants of a wide variety of species. The area over which black walnut trees exert this influence is coincidental with the spread of the root system of the tree. Davis (7) sought to identify this injurious effect with the compound juglone (5-hydroxynaphthoquinone) which is found in both the aerial parts of the plant and the roots. He showed that this compound is toxic to alfalfa and to tomato plants. Davis did not, however, show that juglone is liberated to the soil by roots of black walnut, and he did not establish that the injurious effects of black walnut trees on other plants in the field is due to the liberation of juglone or any other toxic substance. He merely correlated the existence of a toxic substance in the plant with the observed inhibitions carried out by the tree.

A second instance of toxicity owing to plant inhibitors produced by roots was clearly demonstrated in the elegant experiments of H. M. Benedict (2). Benedict worked with a brome grass (*Bromus inermis*). This grass, after growing several years, attains a condition known as "sodbinding" in which the stand begins to thin out and the plants to die back. Benedict suspected that this dying out of old brome grass stands might be due to the accumulation of toxic substances produced by brome grass roots. He showed that dried roots of brome grass are inhibitory, even in small amounts, to growth of brome grass seedlings. This was demonstrated, for example, in nutrient culture or in sand culture by taking a nutrient solution leached through an old culture of brome grass and supplying this leached nutrient to seedlings. It was also demonstrated by the incorporation of dried brome grass roots in sand in which further brome grass seedlings were grown. Benedict did not, however, show that any toxic substances from brome grass roots actually accumulate in sod in old brome grass stands to the extent necessary for inhibition. In short, while he established the presence of a toxic substance in brome grass roots, he did not establish that root inhibition is active under field conditions. Meyers and Anderson (18) have sought to identify sod binding of brome grass with nitrogen deficiency and have shown that application of nitrogen can partially overcome the growth inhibition which results from culture of brome grass in soil which has previously supported brome grass. Their data clearly reinforce the conclusion of Benedict in that well fertilized brome grass soils still give yields much smaller than those obtained from soils in which brome grass has not previously grown.

A third investigation of recent years which bears on this discussion is that of Proebsting and Gilmore (23), which concerns the difficulty of re-establishing peach trees in soil which had previously supported a peach orchard. After ruling out nutritional factors or pathogenic organisms as causative agents in the slow growth of replanted peach trees, Proebsting and Gilmore showed that peach roots when incorporated into sand are inhibitory to the growth of young peach trees. This effect was duplicated by alcohol extracts of peach roots, the alcohol-insoluble residue being non-toxic to peach trees. Here again, while it was clearly demonstrated that the roots of a particular species contain an inhibitor of growth, it was not shown that the substance concerned actually escapes into the soil and is active under field conditions.

Two less well-known investigations concerning plant toxic substances are those of Waks (39) and Bode (3). Waks showed that the roots of *Robinia pseudo-acacia* contain a principle highly inhibitory to other species, and he proposed that this toxin may contribute to the lack of undergrowth in *Robinia* plantings. Bode worked with an entirely different sort of inhibitor, one given off by leaves of *Artemisia Absinthium.* This investigation will be considered later.

EXCRETION OF ORGANIC SUBSTANCES

A matter of general interest to the problem of toxic substances given off by plants is the question of whether or not living roots are capable of giving off organic substances to soil. The idea of active root toxicity supposes that roots may be able to give off to the soil specific organic substances. It is evident that there may be, in addition, another kind of root toxicity, that in which toxic substances may be liberated from dead or dying roots, and it may not always be possible to distinguish between the two kinds of toxicity. In any case it is well established that living roots are able to give off organic substances to the soil or other substrate in which the plants are grown. This was shown by early workers who found that the lower plant acids are excreted by roots; oxalic acid, for example, is given off by *Oxalis* (9). Legume roots also excrete sizeable quantities of organic acids and of amino acids, as has been shown by Virtanen *et al.* (37, 38). These instances are the best authenticated cases of excretion of organic materials by roots. On this basis it would seem entirely possible that organic substances in the soil could indeed arise as excretions from living roots. It has not as yet been entirely satisfactorily established, however, that in any particular case of a suspected root toxin the substance is actually excreted by living roots.

Final demonstration of production by a given plant of a substance inhibitory to the same or other species can be only by isolation of the compound in pure form and demonstration that the substance isolated is actually the agent by which the inhibition is achieved. In the two cases which will next be considered, both of these criteria have been met.

THE MOST RECENT INVESTIGATIONS

GUAYULE. Early in 1940 the plant physiology group at the California Institute of Technology instituted a program of research on growth and rubber formation by guayule. During visits to the guayule plantings at Salinas observations were made which suggested that guayule may under some circumstances show evidence of severe root interaction. Thus in a closely planted nursery the edge rows of plants are much larger than the center rows. This did not appear to be due to water or nutrient competition, since the differences in growth were not eliminated by heavy watering or by heavy applications of fertilizer. It was also observed that when plants grew adjacent to one another the roots of one plant did not intermingle with those of a second but grew out into areas not explored by other guayule roots. Experiments were therefore set up to determine whether it might be possible that guayule gives off a growth inhibitor. The first of these experiments (5) involved leaching of nutrient through sand cultures of one-year-old guayule plants. This leached nutrient was then supplied to younger plants also grown in sand culture. It was found that whereas guayule plants fed with fresh unleached nutrient grew rapidly, plants supplied with nutrient which had been leached through the sand containing an old guayule plant were greatly inhibited in growth. This effect was not due to removal of nutrient by the older guayule plant, as was shown by analysis of the nutrient solution. In addition the nutrient solution leached through guayule plants was entirely inactive in inhibiting the growth of tomato plants. Similarly, nutrient solution leached through cultures of actively growing tomato plants was inactive in inhibiting the growth of guayule.

A second method of demonstrating the production of toxic substances by guayule sand cultures was based on recirculation by a continuous drip method through either cultures containing seedlings only or through cultures in which seedlings were grown in one pot and eight-month-old plants grown in a second pot. Thus in one series the nutrient solution was recirculated over the seedling plants only, while in a second series the nutrient solution was circulated through a more mature plant and then through a seedling plant. The nutrient solutions in both cases were adjusted at regular intervals to make up for the amounts of nutrients removed from solution. It was found that the presence of an older plant in the recirculation system very considerably inhibited the growth of the guayule seedlings.

In a third experiment seedling guayule plants were grown under larger guayule plants. In nature seedling plants are seldom found under mature guayule plants but are found growing under other kinds of shrubs, as is general with desert plants. In this experiment use was made of one-year-old guayule plants growing in sand culture and pots. Two guayule seedlings were planted in the sand adjacent to the guayule plant. In addition a small excavation in the sand was made and a glass jar inserted into this hole. The glass jar was then filled with fresh sand and two other guayule seedlings planted in it. Thus the seedlings in the glass jar received the same shading effects as those received by the plants grown in sand directly under the guayule plant. They were not, however, subjected to any possible inhibitory influences which might be contained in the sand around the guayule plant. Seedlings grown under guayule plants showed a high mortality and grew at a slow rate, whereas those grown under the same conditions but in a separate glass jar showed good growth and a lower mortality.

Isolation of the toxic substance given off by guayule roots was made possible by a test for toxicity which was based upon the growth of guayule seedlings in nutrient solution. The fractions to be tested for toxicity were added directly to the nutrient solution in which the seedlings were grown. The source of toxic material for the initial experiments was nutrient solution which had been recirculated over older plants. For this purpose guayule cultures were arranged in a greenhouse in such a fashion that nutrient solution was continuously dripped over them. The nutrient solution which drained through each crock was then pumped back and recirculated. 240 plants were recirculated with 60 gallons of nutrient solution and the recirculation continued for 30 days. At the end of this time the recirculated nutrient solution was concentrated and subjected to fractionation.

Nutrient solution which had been recirculated in the above fashion over guayule plants for 30 days was found to be inhibitory to the growth of seedlings in the standard seedling toxicity assay. Thus the nutrient solution used supported only approximately 50% of the growth of control plants grown in nutrient solution which had not been recirculated over older plants. The toxicity of this material was heat-stable, but was destroyed by ashing. It was, futhermore, an ether-soluble organic acid. These observations show clearly that nutrient solution recirculated over guayule plants does contain an inhibitor of guayule growth and that this inhibitor is an organic compound.

Numerous experiments were made in an attempt to isolate the compound from this source. They failed, however, because of the fact that 60 gallons of nutrient solution obtainable only by investment of much labor yielded about 80 milligrams of highly active concentrate which was not in pure form. Isolation of toxic material from other sources was therefore attempted, and success was obtained in the following way. Guayule plants taken from a nursery and possessing long straight roots were superficially washed, then packed in crocks, and distilled water placed over them. The water was allowed to leach the roots for one day and was then drained off and concentrated. 20,000 roots of guayule plants yielded in this fashion about 460 grams of total solids. From 460 grams of total solids, which were highly toxic to the growth of guayule seedlings, about 17 grams of ether-soluble organic acids were obtained. These 17 grams in turn yielded in separate experiments approximately 1.6 grams of a crystalline toxic material. This material was shown to be identical with trans-cinnamic acid (5). Trans-cinnamic acid is highly toxic to guayule seedlings, about 50 milligrams per liter causing one-half inhibition of growth in height in the standard toxicity assay and as little as one milligram per liter giving significant growth inhibition.

Cinnamic acid is a normal constituent of the guayule plant as well as of other plants which contain essential oils or resins. In guayule cinnamic acid is found both in the free and in the combined form, the combined form being a constituent of the resin, partheniol cinnamate. Since the compound obtained from washed roots is a normal constituent of the plant, it seems unlikely that it arises secondarily from the bacterial decomposition of dead roots, but it may represent an actual excretion of the root. It might be mentioned that cinnamic acid appears to be selective in its toxicity. Thus guayule seedlings were found to be at least a hundred times as sensitive to cinnamic acid as tomato seedlings. Cinnamic acid is toxic to the growth of guayule plants even in soil cultures, the incorporation of ten milligrams of cinnamic acid in 1,500 grams of soil, or a concentration of less than one part in 100,000, exerting a significant effect in depressing the growth of the plants over a period of six weeks (4). Cinnamic acid is unstable, however, in soil and disappears relatively rapidly, as shown by experiments in which the toxicity of soil containing varying amounts of added cinnamic acid was tested after varying intervals of time. In no case did a detectable amount of cinnamic acid remain after eight weeks, even in cases where as much as one gram of cinnamic acid had been added to 1,500 grams of soil. Experiments undertaken to determine the nature of this disappearance showed that cinnamic acid is stable in soil which has been sterilized by autoclaving. On the other hand it rapidly disappears from soil which is not sterilized and which is incubated at ordinary temperatures and with a water content approximating field capacity. Thus when one-tenth milligram of cinnamic acid was incorporated per gram of soil and the soil then incubated, all toxicity disappeared within two days. When ten times as high a concentration of cinnamic acid was incorporated into soil, all of the added toxicity disappeared within 14 days. The conclusion then would appear to be that cinnamic acid is destroyed in soil, probably by activity of microorganisms, since this disappearance does not take place in sterilized soil. Accumulation of cinnamic acid under field conditions would be expected to be small or negligible, therefore, in soils which contain organisms capable of carrying out this destruction.

A series of investigations were made of the possible content of growth-inhibiting substances, such as cinnamic acid, in soils of different types which had supported the growth of guayule plants in the field. In no case was any toxicity found in these field soils, even though guayule plants had been growing in them for periods up to eight years. Toxicity was found, however, in soil from pot cultures which had supported growth of guayule plants for periods of one to two years and in which severe pot binding had occurred.

In conclusion it may be stated that no soil accumulation of toxic substances could be demonstrated in any field planting where guayule had been shown to grow successfully. The investigation has established that guayule does give off, or lose to the surrounding medium, a toxic compound. Under conditions of good agricultural practice, however, this substance does not appear to accumulate and it may be destroyed as rapidly as it is produced. In any case the production of the toxic substance appears to be of significance only in pot culture or perhaps in nurseries where plants are grown in crowded conditions for long periods of time.

ENCELIA. A second instance of a toxic substance given off by a higher plant is that of *Encelia farinosa,* a desert shrub of the family Compositae. *Encelia,* in contrast to most desert shrubs, does not harbor a growth of annuals under its branches. This fact is of particular interest because shrubs of closely comparable size and habit, such as *Franseria dumosa,* do harbor considerable populations of desert annuals (40). The phenomenon might be due to any number of interactions between *Encelia* and associated species. Experiments (11) show, however, that the lack of associated plant growth with *Encelia* may be due to a toxic substance given off by *Encelia* plants. In preliminary experiments *Encelia* was grown in the greenhouse in sand culture, and the possibility of toxic substances arising from its roots was investigated by methods similar to those used with guayule. No evidence of any toxic substance from the roots could be found. The effects of *Encelia* leaves on the growth of other plants was then investigated. This was done in a wide variety of ways. In one type of experiment, leaves were scraped up from sand under the *Encelia* plant in its natural habitat. These leaves were then brought to a laboratory and placed as a mulch on the surface of sand in which a tomato plant was growing. It was found that as little as ten grams of leaves per tomato culture caused severe retardation of growth, while ten to 20 grams of leaves frequently killed the tomato plant. If the leaves were incorporated in the sand the effect was even greater. There is a certain selectivity in the action of *Encelia* leaves in inhibiting plant growth, since tomato, pepper and corn are greatly inhibited, while *Encelia* itself, barley, oats and sunflower appear to be but little affected. In a further study of this problem, use was made of a toxicity assay involving the growth of tomato seedlings in nutrient solution, an assay essentially similar to that used for the guayule toxic substance. The materials to be tested were added to the nutrient solution and in this case were generally so toxic that it was necessary merely to inspect the test plants after one day and determine which ones had been killed and which ones remained alive.

The toxic substance of *Encelia* leaves was found to be extractable with ether, the ether extracts being highly toxic to tomato seedlings, while the residue left after ether extraction was essentially non-toxic. An extract of 50 milligrams of *Encelia* was found to kill one half of the test tomato plants within one day, while by contrast an extract of 100 milligrams of tomato leaves was without effect. The inhibitor contained in *Encelia* leaves was non-volatile, heat-stable, and appeared to be a neutral compound. The active material involved was isolated from dried *Encelia* leaves by successive fractionations with benzene, water, benzene petroleum ether, and recrystallization from ether, the leaves yielding about 0.5 milligram of pure crystalline inhibitor per original gram of leaves. There is undoubtedly several times this amount present, since the mother liquors from which the material was crystallized contained additional amounts of the substance. Inhibition of growth in the tomato seedling test was brought about by concentrations of the order of 20 milligrams per liter, half inhibition being caused by a concentration of 50 milligrams per liter. At a concentration of 250 milligrams per liter all of the test plants were killed within one day. The toxic substance isolated from *Encelia* leaves was shown to have the structure 3-acetyl-6-methoxybenzaldehyde, a compound not previously known either in nature or to organic chemistry. The structure of this compound was confirmed by synthesis and the synthetic compound shown to have the full activity of the natural material (12).

Inhibition of germination and growth of seeds and seedlings of normally shrub associated species has been achieved both in the field and in the laboratory by placing a mulch of *Encelia* leaves over the substratum containing such seeds. It is known also that the leaves which fall from the *Encelia* plant to the ground under the *Encelia* shrub retain their toxicity toward tomato and other sensitive plants for at least a year or more, and that this toxicity may be leached into the soil by water, as for example by rainfall. There seems to be no escape from the conclusion that under natural conditions the growth of susceptible species will be inhibited by the *Encelia* substance, and the possibility that the lack of desert associated annuals with *Encelia* is owing to the leaf produced toxic substance would seem to merit attention.

OTHER RECENT INVESTIGATIONS

Another case of plant induced toxicity has been described by Bode (3) and later discussed in detail by Funke (10). Bode worked with *Artemisia Absinthium* which, like *Encelia,* contains in its leaves an inhibitor of the growth of other species. The active substance was tentatively indentified by Bode as absinthin, a glucoside of unknown composition which is secreted by glandular hairs of the leaves of *Artemisia Absinthium.* The toxic material is washed off by rainfall onto the ground around the *Artemisia* plant and may then severely stunt other species, for example, *Salvia sclarea, Leviticum, Nepeta* and *Carum,* this action extending to distances as great as a meter from the *Artemisia* plant. *Artemisia vulgaris,* a shrubby species of growth and habit comparable to *Artemisia Absinthium,* but which does not contain absinthin, exerts no such inhibition. Experiments done with field plantings by Bode and by Funke established that *Artemisia Absinthium* under these conditions also exerts an unfavorable effect upon a wide variety of other species. Thus the toxicity of *Artemisia Absinthium* toward other plants is reflected in the weed vegetation in fields of this crop. According to Funke (10), weeds resistant to *Artemisia* toxin are favored (*Stellaria, Datura*) over those inhibited by the toxin (*Senecio*).

SUMMARY

The alleged existence of plant toxic substances would seem at present to be somewhat better supported than it has been in the past. The evidence that such substances affecting the growth of other plants do exist is best in the case of the two leaf-produced toxic substances of *Encelia* and of *Artemisia*. Good evidence is also available that guayule roots may give off a toxic substance. Substances inhibitory to growth have been shown to be contained, furthermore, in the roots of peach, brome grass, *Robinia* and black walnut, but whether these substances are given off to the soil, and whether they exert their effect in nature is at present unknown. Complete evidence that toxic substances are effective under normal or field conditions is available at present only in respect to *Artemisia Absinthium* and *Encelia farinosa*. In guayule, although toxic substances are produced, they are rapidly destroyed by the soil under conditions of guayule cultivation. The guayule substance would not then appear to be of economic importance.

Of more importance is the question of the extent to which specific toxic substances produced by certain species may be responsible for ecological phenomena, such as the composition of plant communities or the sequence of particular species in a succession. The observations on *Encelia* suggest that the toxic substance produced by it may be of great importance in determining the floristic composition surrounding this plant. The same is true of the observations on *Artemisia* in cultivation. Preliminary unpublished observations of the author suggest that numerous species of desert shrubs may, like *Encelia,* produce substances toxic to one or more other species. Association or non-association of different species as a result of specific chemical compounds secreted by them may therefore be of not uncommon occurrence, and it will be of great interest to determine the exact importance of this mode of plant interaction in other cases.

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DEVELOPMENT AND STRUCTURE OF THE PHLOEM TISSUE. II[1]

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INTRODUCTION

The new literature on the development and structure of the phloem is not voluminous but it has caused some important changes in the terminology and concepts regarding this tissue. Some investigations have clarified details in the ontogeny of the phloem and have contributed toward the understanding of its seasonal changes. The phylogenetic aspects of the sieve-tube structure in the monocotyledons have been thoroughly investigated and, concomitantly, some advance made toward the establishment of a uniform terminology regarding the conducting elements. The controversial topic of the structure of the sieve element as related to its function was subjected to experimental studies and was much discussed. As a result, comprehension of the essential characteristics of the protoplast of a sieve element has been deepened.

The present review has a three-fold purpose: to sum up the work that has been done on the phloem tissue during the past ten years; to stabilize, as far as possible, the terms used with reference to the tissue and its components; and to bring up to date the concepts on the relation between the structure of the sieve element and its function. It has a somewhat different topical arrangement than the previous review, and the topics to which no new information has been added are entirely omitted. Such topics are: historical data; ontogentic transformations of the sieve element; characteristics of the parenchymatous elements of the phloem; interconnection of phloem cells; and the phloem in vein endings of leaves.[2]

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[1] Supplement to article in The Botanical Review **5:** 373-432. 1939.

[2] There is, however, a report (86*a*) on the quantitative aspect of phloem distribution in the veins of leaves. According to the investigators, six dicotyledonous species contained only parenchyma in the phloem of minor veins, and these veins composed 95 per cent of the total vein length of the blade.

The studies on the relation between the function and structure of the sieve element are given special attention. By considering the methods employed by the investigators, as well as their conclusions, a critical evaluation of the various concepts on the nature of the sieve-element protoplast is attempted.

THE CONDUCTING ELEMENTS

CLASSIFICATION. Since the appearance of the first review on the phloem tissue in this journal (33), a practical terminology has been proposed by Cheadle and Whitford (15) for the designation of the characteristic conducting elements of the phloem. According to this terminology, the elements in question bear "sieve areas" on their walls, that is, pit-like[3] recesses where pores with connecting strands are grouped. The sieve areas are variously distributed on the wall and may be unlike on different parts of them. A wall or part of a wall bearing one or more highly specialized sieve areas, that is, areas with particularly conspicuous connecting strands, is referred to as "sieve plate". A parallel term for the xylem is "perforation plate", denoting the area of the vessel wall which is involved in the coalescence of two members of a vessel (18). If the sieve plate shows a uniform distribution of pores (one sieve area), it is called a "simple sieve plate"; if the pores are in clusters arranged in scalariform, reticulate or any other manner (several sieve areas), the sieve plate is "compound". Most commonly it is the end (or terminal) wall, transverse or inclined, that is differentiated as a sieve plate, but such structures may be found on longitudinal (or lateral) walls as well.

Conducting elements having sieve plates are usually disposed in long series end to end. These series are the "sieve tubes". One unit of such a series is a "sieve-tube member" (15), or a "sieve-tube element". Thus a sieve-tube member may be characterized as a cell "in which certain sieve areas are more highly specialized than others, the former being largely localized on end walls to form the sieve plates" (15). If "the sieve areas of all walls are of the same degree of specialization" and no wall parts can be conveniently distinguished from others as sieve plates, the conducting element constitutes a "sieve cell" (15). The sieve cells are commonly elongated elements with tapering ends and are not arranged in longitudinal series like the sieve-tube members. Cheadle and Whitford (15) include in the definitions of the conducting elements the absence of nuclei and the deposition of callose around each connecting strand of a sieve area.

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[3] According to the Committee on Nomenclature (18), "pit" designates a recess in a secondary wall only, and "primary pit field" a thin area of the primary wall and intercellular material. Such a strict distinction between the terms "primary pit field" and "pit" requires an equally accurate recognition of primary and secondary walls. A precise diagnosis of the nature of walls is often beyond the scope of an anatomical study, and then the investigator is left without a term for the thin areas in the walls. As in the previous review (33), the present writer again uses "pit" to identify a recess in either a primary or a secondary wall.

It is of interest to recall at this point that Hartig (58), who discovered the sieve element, was aware of the difference between sieve cells and sieve tubes, and used the term "Siebfaser" to designate the former.

The terminology just considered does not suggest a name that would include both the sieve-tube members and the sieve cells. In this review the two types of cells will be united under the term "sieve element" which was recently adopted by Eames and MacDaniels (30, p. 103). It is admittedly not an entirely satisfactory term because it can be confused with "sieve-tube element", but it has the advantage of utilizing a well known word, "sieve", which is rather universally associated with the conducting elements of the phloem, and it is not a new term.

The terminology given above parallels that which is used with reference to the conducting elements in the xylem. While the phloem has "sieve elements", which may be segregated into "sieve cells" and "sieve-tube members" (or "sieve-tube elements"), in the xylem there are "tracheary elements" (50) of two types, the "tracheids" and the "vessel members" (or "vessel elements"). Whereas sieve-tube members are combined into "sieve tubes", vessel members form "vessels". In both groups of terms the degree of specialization of the characteristic wall structures determines the final classification. The higher degree of specialization in the sieve elements is reportedly expressed in the enlargement of the connecting strands and, consequently, also in the increase in diameter of pores and of the callose cylinders encasing the strands. In the tracheary elements specialization has led to the removal of parts of wall and the establishment of direct continuity between the individual cells. The sieve cell is regarded as more primitive than the sieve-tube element; similarly the tracheid is interpreted as a more ancient type of cell than the vessel element.

Sieve-tube members are usually associated with specialized parenchyma cells, the "companion cells", which are ontogenetically related to the sieve-tube members. Sieve cells of gymnosperms and still lower vascular plants are not known to be accompanied by similar cells, except that in the gymnosperms the "albuminous cells" of the rays appear to be physiologically associated with the sieve cells. According to Barghoorn (*7a*), "albuminous cell" should be abandoned and replaced by "erect phloem ray cell" because the former term implies a protoplasmic peculiarity as yet unsubstantiated. Barghoorn thinks the dense staining of the albuminous cells is related to their narrow diameters and to the resulting retention of whole protoplasts in sections, in which larger cells, in contrast, are cut open and lose part of their cytoplasm. He also stresses that, contrary to a common concept, the albuminous cells do contain starch. As was pointed out in the previous review (33, p. 410), the albuminous cells have been reported occurring not only in the rays of gymnosperms, but also in the vertical system, where, except for the appearance of their protoplasts, they resemble the phloem parenchyma cells. Furthermore the presence of starch during certain times in the life of the albuminous cells was recognized by their discoverer, Strasburger. Although the term "albuminous cell" is unsatisfactory, the abandonment of the concept is not justified by the available information.

SIEVE AREAS. The sieve areas are comparable to pits in living cells in that they constitute thin places in the wall, in which the strands connecting the protoplasts of the superposed or adjacent cells are aggregated. Since the exact nature of the connecting strands, that is, whether they are entirely similar to plasmodesmata or not, is not yet known (see 33 and part of this review concerned with the relation between the structure and function of the sieve element), the present reviewer prefers the term "connecting strands" for the designation of these structures.

Sometimes a sieve area is clearly circumscribed and is uniformly thin throughout its extent; or it appears to be broken up into smaller units separated from each other by slightly thicker wall portions, not so thick, however, as the unpitted parts of the wall.

Even single strands may be surrounded by thickened wall portions (see example in 44). The various patterns assumed by the sieve areas are, of course, related to the method of cellulose deposition in primary walls. As is well known, the deposition of fibrils of cellulose in such walls is not uniform throughout, and some fibrils may subdivide the original primordial pit area into smaller ones, thus creating a field of pits, which all together are recessed within the unpitted part of the wall (e.g., 7, 97). The question then, whether a whole sieve area, or part of it, or one connecting strand, corresponds to a pit (see 33), has no simple answer. Varied situations occur and it is possible that in plants with particularly large strands an entire pit with several plasmodesmata is converted into one connecting strand. Still other variations are possible, such, for example, as the inclusion of several connecting strands in one callus cylinder—a pattern reportedly characteristic of gymnosperms (33).

The combination of all pit-like wall structures of the sieve elements under one term, "sieve area", as proposed by Cheadle and Whitford (15), is to be desired, firstly because the various sieve areas appear to be fundamentally similar in their make up, and, secondly, because sieve areas of distinctly different degrees of differentiation may be connected by transitional structures in one and the same cell (33, 44).

The view on the spatial relation of callose and cellulose in the sieve areas has remained largely unchanged in the works on phloem. Each connecting strand is at first enclosed in a callus cylinder, but later large masses of callose are deposited on the sieve area, the connecting strands becoming longer and thinner in the process. If the deposition of the callose is associated with a complete cessation of function of the sieve element, the accumulations on the sieve plate are called "definitive callus"; the callus masses which occur during a temporary break in activity, as in *Vitis,* may be termed "provisional" or "dormancy callus" (33, 44).

The implausible assumption that the callose is derived from the sieve-tube slime has again appeared in the phloem literature (95).

THICKENING OF LATERAL WALLS. The occurrence of thick, apparently hydrated walls in differentiating and mature sieve elements has been observed in many plants of different taxonomic groups (33). These walls are commonly designated as "nacré", a term taken from the French literature (33). No satisfactory substitute term has been as yet proposed, principally because information on the nature of this wall is still rather fragmentary.

It has been recorded that the nacré thickening is transitory, that it loses water and becomes thinner as the sieve element ages (33). This reduction in thickness is not necessarily a constant character. Thus, according to Sterling (110), the nacré thickening of protophloem cells in *Sequoia* persists until the cells are obliterated.

Schneider (100) recently confirmed the cellulose nature of the nacré thickening, using peach and cherry phloem. Crafts (24, 25) and Sterling (110), studying gymnosperms, found it to be doubly refractive.

Some plants appear to lack the special thickening of sieve-element walls. For example, it is not characteristic of *Vitis* sieve tubes, at least not of those in the secondary phloem (44).

SIEVE ELEMENTS IN DIFFERENT GROUPS OF PLANTS

The revised terminology regarding the conducting elements of the phloem calls for a reexamination of these structures in the different groups of plants, especially in those bearing no seeds. Although descriptions of sieve elements in the lower vascular plants have been given, they were written mostly by authors who did not have in mind the distinction between sieve cells and sieve-tube elements. Furthermore, the evolutionary trends in the structure of the phloem and its elements are not yet adequately known in the vascular plants in general. The following attempt at classifying the sieve elements in different groups of plants is therefore entirely tentative.

DICOTYLEDONS. The angiosperms generally have sieve tubes in both the primary and the secondary phloem. In dicotyledons the primary sieve-tube elements apparently vary less in structure than the secondary. The former usually have transverse or slightly inclined end walls bearing simple sieve plates. The secondary sieve-tube elements range from types more or less resembling the sieve cells of gymnosperms, as in *Pyrus, Sambucus* (74) and *Sorbus* (62), to those with transverse end walls bearing highly differentiated simple sieve plates (e.g., herbaceous species, *Robinia*). On the basis of the very meager comparative data on the phloem of dicotyledons (60–62, 74, 83), the concept has been formulated that the phylogenetic specialization of the sieve elements in this group of plants involved a shortening of individual cells; a decrease in inclination of end walls; a decrease in elaboration of sieve areas on longitudinal walls; and an increase in the size of pores in the sieve areas of the sieve plates. This concept obviously suggests a parallelism between the evolution of the sieve elements and that of the tracheary elements in the xylem.

MONOCOTYLEDONS. The thorough survey of the metaphloem in monocotyledons by Cheadle and associate (14–16) strengthens the concept of evolution of the sieve element just outlined for the dicotyledons. In 219 species in 158 genera of 33 families of monocotyledons, only sieve tubes were found in the metaphloem. The trends in the specialization of these elements may be summed up as follows: progressive localization of highly specialized sieve areas on the end walls; gradual change from very oblique to transversely placed end walls; gradual change from compound to simple sieve plate; and progressive decrease in conspicuousness of the sieve areas on the side walls.

Authors (14, 15) generally found the most highly evolved sieve tubes in leaves, in inflorescence axes, corms and rhizomes; the less highly specialized ones in the roots. The sieve tubes of the aerial stems occupy an intermediate position. Such course of evolution in the phloem of the plant as a whole is the reverse of that in the xylem; the vessels specialize first in the roots and then upwardly throughout the shoot (16).

The degree of specialization of the sieve elements, as expressed in the position of the end wall, is correlated with the specialization of the plant or organ as a whole (15). The sieve elements in the families Palmae, Pandanaceae, Dioscoreaceae, Smilacaceae, and in some of the Liliaceae have inclined end walls in stems and roots. Similar sieve-tube end walls occur in the roots of the Agavaceae, Zingiberaceae, and in many Liliaceae. All these families or genera are perennial and commonly woody in nature. The sieve elements in plants with corms and bulbs (e.g., Amaryllidaceae), in hydrophytes, and apparently in most of the sedges and grasses have transverse end walls. The same type of wall is characteristic also of the annuals, including annual roots, and of the parts of perennial plants that are annual in nature.

With regard to the phloem of the monocotyledons, the structures described by Mason (77) in *Dioscorea* have been given renewed consideration. According to Mason, at each node of this plant glomeruli composed of peculiar parenchyma cells intervene between the sieve-tube strands of the superposed internodes. Roeckl (91), however, found evidence that these glomeruli might be composed of sieve elements and that therefore the phloem does not appear to be interrupted at the nodes by non-typical phloem tissue.

GYMNOSPERMS. The secondary sieve elements of gymnosperms are sieve cells. They are elongated and tapering in shape and lack sieve plates. The primary sieve elements in this group of plants are probably sieve cells also (24, 25, 56, 110, 111), although in the leaves of the conifers these elements are described as having nearly transverse end walls with sieve areas on the latter only. The nature of the very first phloem elements seems to be uncertain. They show no distinct sieve areas and may contain dense cytoplasm (56, 110, 111). Perhaps these elements are of the type described by Chauveaud (see 33) as "precursory phloem" which he found below the cotyledons in gymnosperm seedlings. Gunckel and Wetmore (56), who recognized such imperfectly developed phloem elements in the protophloem of the shoot system of *Ginkgo,* prefer to classify them as "sieve cells" because they are connected by transitional types of cells with the later-formed primary sieve cells. Bouvrain (11*a, b*) reports having found precursory phloem in the seedling roots of certain dicotyledons, but furnishes no satisfactory proof that the structures in question are not ordinary protophloem sieve tubes.

LOWER VASCULAR PLANTS. The sieve elements of the non-seed-bearing vascular plants would probably fall mostly into the class of sieve cells (see 33). It is of interest that Russow (93) distinguished between sieve cells (which he called "Siebröhren") and sieve tubes ("Siebgefässe") in these plants and stated that the various vascular cryptogams have mostly long spindle-shaped sieve elements, except *Equisetum* and the Ophioglossaceae in which these elements are prismatic with horizontal or somewhat inclined end walls, and occur in continuous files. Dippel (29), who apparently discovered the sieve elements in the cryptogams, also noted the horizontal end walls in the phloem elements of

Equisetum. It is possible, of course, that despite their shape these sieve elements have no sieve plates in the sense of Cheadle and Whitford (15) and should be called "sieve cells", as was done by Golub and Wetmore.

According to the somewhat detailed study and drawings of West and Takeda (124), the sieve elements of *Isoetes* resemble primary sieve-tube elements of the dicotyledons in their shape. They have transverse or somewhat inclined end walls. Sieve areas occur on all walls, but on the end walls the callus masses develop more prominently than on the lateral walls.

These few references to the phloem of the lower vascular plants should suffice to illustrate the fragmentary nature of the data about the sieve elements in these groups.

ALGAE. Since the sieve elements of Laminariaceae are so similar to those of herbaceous angiosperms, they probably should be called "sieve-tube elements". A new study of these structures by Smith (109) did not contribute toward their proper classification. In fact, disagreeing with previous workers (see 33), Smith identified sieve elements only in *Nereocystis*. He used an unusual method for identifying sieve tubes: instead of looking for connecting strands and the associated callus, he strived to see holes in the sieve plates, a rather problematical undertaking when the connecting strands, and consequently also the pores, are as fine as those in the Laminariaceae. Smith also reports that nuclei are present in the sieve tubes of *Nereocystis*.

CONCLUSION. Thus the presently available information on the types of sieve elements in different groups of plants suggests that sieve tubes are characteristic of the primary and secondary phloem of angiosperms and also occur in certain brown algae; that gymnosperms and the non-seed-bearing vascular plants typically possess sieve cells; and that the lower angiosperms have sieve elements which resemble sieve cells or are perhaps transitional between sieve cells and sieve-tube elements.

THE PROTOPLAST OF THE SIEVE ELEMENT

THE ENUCLEATE CONDITION. The protoplast of the sieve element has been intensively studied by investigators interested in the problem of translocation of organic solutes. Its cytoplasm has been variously interpreted as showing properties of normal living substance or as being denatured and having lost its property of differential permeability. These concepts are examined in detail in the part of this review dealing with the relation between structure and function of the sieve element.

The enucleate condition of the sieve-element protoplast is a well known characteristic (see 33) and has recently been confirmed for a large number of angiosperms not previously investigated from this viewpoint (95). The absence of a discrete nucleus from a fully differentiated sieve element raises the question of appropriateness of the name "protoplast" for the contents of such an element. "Cytoplasm" alone does not properly describe these contents because in a certain sense they are organized as a vital unit, somewhat like a protoplast: a parietal layer of cytoplasm encloses a vacuole (often rich in colloidal matter, the slime) and this unit is able to perform some vital functions after loss of the nucleus. The present reviewer favors the continued application of the term "protoplast" with reference to this enucleate unit of living matter with its non-living inclusions. The term "cytoplast", which is present in biological literature, seems to be less appropriate because it denotes the cytoplasmic part of a cell as distinguished from the nucleus and does not appear to imply enucleate condition.

A discussion of the sieve-element protoplast usually brings up the disputed point about the stage of development of this protoplast that coincides with the functioning state of the element. The majority of workers consider that the functioning period of the element begins after disintegration of the nucleus and dispersal of the precursors of the slime, the "slime bodies". At this stage the connecting strands are most conspicuous because of their thickness and density of staining, and are associated with only small amounts of callose. The evidence that such elements are in a functioning state is, of course, indirect. The developmental stages are passed rather rapidly, and the sieve elements are without nuclei during the longest time of their existence as intact cells.

The occurrence of dormancy and reactivation in grape phloem offers a unique opportunity of testing the anatomist's notion of a functioning sieve tube (44). The phloem of *Vitis* functions for more than one season and passes, in the aerial parts of the plant, through a period of dormancy during the winter. The dormant sieve tubes deposit heavy masses of callose on the sieve plates, but these are removed during reactivation in the spring. The reactivated sieve tubes assume the same characteristics as the recently differentiated elements of the new season; that is, they show no nuclei, but have cytoplasm, slime, sieve-tube plastids and prominent connecting strands enclosed in relatively thin callose cylinders. These characteristics are exhibited by the phloem throughout the period of active growth of the plant, first in the reactivated, then in the newly differentiated sieve tubes. The interpretation of the enucleate sieve-tube elements as functioning seems well justified on the basis of the behavior of grape phloem.

SLIME BODIES AND SLIME. The occurrence of rather dense colloidal structures, the "slime bodies", in the cytoplasm of young sieve elements, and their eventual dispersal into the slime of a maturing element has been previously noted in many dicotyledons (see 33). Recently many additional dicotyledonous species have been shown to have slime bodies (95). The relative persistence of these bodies in the sieve tubes of certain Leguminosae has also been confirmed (95). Not all dicotyledons have equally large amounts of slime. In many tree species the sieve-tube protoplasts are very thin (92). The monocotyledons, the gymnosperms and the still lower vascular plants, in which slime bodies have not been recorded, have particularly thin sap with only small amounts of slime in their sieve elements (1, 33).

The slime bodies and slime are usually interpreted as being proteinaceous in nature. Recently Rouschal (92) reported having confirmed that this material is protein. Salmon (95), however, asserts that slime and slime bodies also contain lipoids, and calls the bodies "lipido-proteinaceous corpuscules" and the slime "lipido-proteinaceous accumulations". There appears to be no reference other than that of Salmon, which associates lipoids with the sieve-element slime. Lipoidal material was reported in phloem by Maugini (79), but not specifically in sieve tubes. Schmidt (98) and Rouschal (92) found none or very little fatty material in differentiated sieve elements.

The slime is assumed to be in dispersed or suspended state in the vacuole, but if the sieve element is cut, the slime accumulates on one or both sieve plates and forms here the so-called "slime plug". Recently Salmon (95) expressed the antiquated view that slime is naturally located on the sieve plate and she interpreted this accumulation as plasma membrane.[4] According to Abbe and Crafts (1), the slime is transitory in nature and soon disappears from the sieve elements. Studies on phloem, which functions for more than one season (such as *Vitis* phloem), clearly show that the slime is relatively persistent and occurs in the sieve tubes during the first and in the subsequent season or seasons of their function. This observation agrees with an old conclusion of Fischer (48) that the presence of slime and the ability to form slime plugs are associated with the functioning state of the sieve tubes, while the disappearance of this substance indicates approaching obliteration.

In a rather limited study of sieve-tube protoplasts based on fixed material, Pigneur (86) found that the slime bodies of *Cucurbita* do not disperse but fuse into one mass. Such fusion of slime in *Cucurbita*—sometimes before dispersal in the vacuole, sometimes simply as a result of coagulation (especially often in fixed material)—had been previously reported in the literature (see 33). The structures, which Pigneur (86) interprets as slime bodies in *Athurium* and pictures as coalescing into small masses and threads, are very small and of a more definite shape than slime bodies usually are.

In *Vitis vinifera* slime bodies have been noted not only in the sieve tubes but also in the companion cells (42, 44). They disperse in the companion-cell protoplasts at about the time when the sieve-tube slime bodies break down. In the companion cell the dispersed slime appears to remain in the cytoplasm. Although the occurrence of slime bodies in companion cells seems to be unusual, the relative density of the companion-cell protoplast is a commonly noted characteristic. This density is a feature that is acquired during differentiation of the companion cell. Possibly, even if slime bodies do not appear as discrete structures in the companion cells, the same kind of substance develops in them in a more or less dispersed state and imparts the dense appearance to the protoplasts of these cells.

The term "slime" as applied to the colloidal and apparently proteinaceous material of sieve elements has been criticized by Schmidt (98) and Rouschal (92) chiefly because the substance is

[4] See the review of Salmon's paper by K. Esau in Bot. Gaz. **109**: 361-362. 1948.

not a true slime. Since, however, the exact nature and function of this slime is not yet known, phloem investigators are not ready to agree upon a more appropriate and a better-sounding term. (See also 42.)

PLASTIDS AND STARCH. Sieve elements of many species of plants contain plastids, the sieve-cell or sieve-tube plastids, which elaborate a carbohydrate giving a red staining reaction with iodine (33). The carbohydrate is regarded as being related to starch and may be referred to as "sieve-cell" or "sieve-tube starch". The wide distribution of sieve-tube starch in angiosperms has recently been confirmed by Salmon (95).

Apparently starch staining red with iodine is not restricted to sieve elements. Küster (70, p. 380–381) mentions such starch in the endosperm of some Gramineae, in the seed of *Sinapis,* in the root cap of *Allium cepa* and in some other structures and plants. It is supposed to contain much dextrin and amylodextrin. The red algae also develop starch that usually becomes red or reddish violet when treated with iodine and is doubly refractive. According to Fritsch (51, p. 410–411), some evidence suggests that this carbohydrate is allied to but not identical with the starches of higher plants. Guilliermond (55, p. 205) states that the Floridean starch is not formed in plastids and is capable of emerging into the vacuole—a characteristic sometimes ascribed to the starch grains of sieve elements.

The opinions regarding the exact location of plastids and starch grains in the sieve element are tied up with the controversy about the nature of sieve-element cytoplasm. Commonly in sectioned material the starch grains may be seen in Brownian motion in the vacuoles of the sieve tubes. The interpretation of this phenomenon by an early authority on plastids is of interest. Arthur Meyer (80) suggested that when phloem sections were placed in water, the sieve-tube plastids often were destroyed and the starch grains appeared in the cell sap where they showed motion. One should, therefore, according to Meyer, study only those sieve tubes whose contents are not in motion. In such elements the plastids appear near the walls or are piled near the sieve plates.

Among subsequent workers, some explain the presence of starch in the vacuole as resulting from the denatured state of cytoplasm; the boundary between it and the vacuole is supposedly obliterated, the plastids break down and release the starch grains into the vacuole (see 33).

Rouschal (92), however, using special methods that enabled him to prevent the usual displacement of sieve-tube contents in sectioned material (see part on relation between structure and function of sieve element), found that the starch grains occurred within the parietal cytoplasm of mature sieve tubes, although very close to the periphery of the vacuole. By inducing a flow through the sieve tubes (adding glycerine on one side of the section), he easily dislodged the starch grains and observed their emergence into the vacuoles and accumulation at the sieve plates.

Sometimes starch is not characteristically present in the plastids. Thus in sugar-beet the carbohydrate was noted only after much material had been surveyed (34). Ordinarily the sieve-tube plastids of sugar-beet stain like cytoplasmic structures (33). The nature of the plastid-like granules in the sieve elements of monocotyledons and ferns is still uncertain (33, 95). Perhaps they are related to the elaioplasts described in some monocotyledons (45).

EXTRUDED NUCLEOLI. The sieve-tube inclusions mentioned under "unnamed inclusions" on p. 386 of the former review on phloem (33) have now been recognized as extruded nucleoli. The structures were first identified as such by Engard (31) in *Rubus;* however, he interpreted the free nucleoli as slime bodies. Esau (42) has shown that the extruded nucleoli are distinct from the slime bodies and that both kinds of structure occur in the same elements. The extrusion of nucleoli occurs during disintegration of the nuclei, and, while the rest of the nucleus disappears, the nucleoli remain in the sieve-tube elements as long as the latter exist as intact cells. The present writer (42) studied extrusion of nucleoli in *Rubus, Eucalyptus* and *Gossypium.* The globoidal inclusions observed by Crafts (20, 22) in *Casuarina, Melicytus, Populus* and *Tilia,* along with those in *Eucalyptus* and *Gossypium* are undoubtedly extruded nucleoli. These structures are readily identifiable in a photomicrograph of *Tilia* phloem given by Huber (62, p. 203, fig. 24) and in the illustrations of sieve-tube inclusions from *Althaea officinalis, Urtica dioica* and *Castanea vulgaris* in Salmon's (95) paper. The latter author did not determine the origin of the bodies and regarded them as slime bodies. Among the older writers, Lecomte (71) evidently saw extruded nucleoli in *Tilia* and *Rubus* but he called them "albuminous globules". Apparently Fischer (48) too referred to extrusion of nucleoli when he said that in *Urtica* the nucleus seemed to dissolve incompletely, leaving as remnant a small disc-shaped structure.

The extruded nucleoli develop a peculiar external sculpture as soon as they leave the nucleus. They form protrusions of various degrees of fineness. In *Rubus,* for example, the protrusions are coarse and short, in *Eucalyptus* quite long and extremely fine (42).

The significance of the extrusion of nucleoli and of their retention after disintegration of the rest of the nucleus in the sieve tubes of some plants is not understood. The list of plants in which this phenomenon was noted thus far suggests that it occurs in widely separated groups of dicotyledons.

Older reports in the literature on the extrusion of nucleolar material into the cytoplasm concerned only dividing nuclei (73; also literature in 42). Recently, however, this phenomenon was described by Milanez (81) as occurring in the laticiferous tubes of *Hevea* during the disintegration of nuclei. As in the sieve tubes, the nucleolar material in the latex ducts is extruded in the form of one to several globules, successively or simultaneously.

STRUCTURE OF THE SIEVE ELEMENT IN RELATION TO ITS FUNCTION

THE SIEVE ELEMENT AS THE MAIN CONDUCTING STRUCTURE IN THE PHLOEM. Although many researches were concerned with the determination of the channels of migration of solutes and good evidence was assembled that the organic solutes move largely in the phloem, the first direct indication of this movement in the sieve elements was obtained only in 1930 by Schumacher (see 78.) By ingeneous operational procedures, this worker (102) was able to study separately the role of ground parenchyma, xylem and phloem in the removal of nitrogen from darkened leaves of *Pelargonium zonale.* His experiments showed that this movement occurs almost entirely through the phloem. In another experiment, eosin introduced into leaves was found to cause a callousing of sieve plates and an eventual collapse of sieve tubes and companion cells, without visibly injuring the phloem parenchyma. By treating with eosin *Pelargonium* leaves which were connected only by a phloem bridge to the stem, Schumacher (102) succeeded in interrupting the removal of nitrogenous and other substances from the leaves. It seemed that the injury to the sieve tubes by eosin was responsible for the cessation of movement through the phloem. In his later experiment dealing with the movement of fluorescein in the phloem, Schumacher (103, 104) could observe microscopically that the dye was present in the sieve tubes and companion cells for long distances from the place of introduction of it into the plant.

IS THE SIEVE-ELEMENT PROTOPLAST DENATURED? Long before Schumacher carried out his investigations on the role of sieve tubes in translocation, the sieve element was generally considered to be the main conducting structure in the phloem. As was detailed in the previous review on phloem (33), some of the proponents of the mass-flow concept of translocation made determined efforts to correlate the structure and function of the sieve element, and their main conclusion was that the protoplast of this element was in a more or less passive state, particularly as expressed in its loss of the property of semipermeability. These efforts were continued during the last decade.

The investigators who were opposed to the concept of pressure flow in the phloem also renewed their observations on sieve elements in order to prove their idea that the protoplast of a functioning sieve element is active. This double attack upon the problem has brought to light new information on the cytophysiology of the sieve element.

The most consistent defense of the idea of permeability of the sieve-element cytoplasm came from Crafts. The previously reviewed microscopic studies of the phloem by this investigator (see 33) led to a formulation of the following concept (22). In the early ontogenetic stages the sieve element is a normal cell in its physiological responses. During maturation, however, the nucleus disintegrates and disappears; cytoplasmic streaming gradually ceases; the parietal cytoplasm loses its clear inner-phase boundary, and the vacuole its ability to accumulate vital stains; and the protoplast ceases to respond with plasmolysis to treatment with hypertonic solutions. Thus it appears that the cell becomes readily permeable to assimilates in solutions, permitting a free flow of sap through its lumen. In a much later paper Crafts (26) offers a hypothesis on the submicroscopic change in the structure of cytoplasm to explain its permeability in the sieve element. He assumes a straightening of polypeptide chains during the process of denaturing, a loosening of bonds, and a loss of lipoidal substances responsible for semipermeable properties. There is supposed to be also a loss of polar groups and with it of the original staining properties. The lost substances are replaced by water, mostly in free state. Crafts concludes that such cytoplasm would offer little resistance to the flow of solution.

The morphological appearance of this cytoplasm is frequently considered by Crafts. In an early work the sieve-tube cytoplasm of potato is described as poorly delimited from the vacuole and appearing simply as a region of greater density or viscosity than the vacuole (see 33.) In the pine, too, the permeable sieve-cell cytoplasm is found to be very transparent and inconspicuous, though at the same time it is referred to as being tough and fibroid (1). In some other plants such cytoplasm is characterized as ductile, elastic, fibroid, tough and stringy (20, 21, 22, 26). It is found lacking in affinity for plasma stains. According to a personal communication from Dr. A. S. Crafts, the reference to the cytoplasm as being heavily stainable, in his paper on the survey of sieve elements in different groups of plants, 21, page 175, is erroneous. The statement should be "less heavily staining".

The significance of these variations in morphology—variations between a cytoplasm, which is only somewhat more dense than the vacuole, and the tough and fibroid structure—is not obvious from Crafts writings, and it is difficult to picture an elastic, tough and stringy cytoplasm as being poorly delimited from the vacuole.

Huber and Rouschal (65) made somewhat similar permeability and vital-stain-accumulation studies as Crafts and tended to agree with him on the interpretation of the nature of sieve-element cytoplasm. The concept of permeable conducting elements seemed to offer the best explanation of the mechanics of mass flow in the phloem. Münch (85), too, found this concept most useful, and in his last paper dealing with translocation emphasized that mass flow was determined primarily by two conditions: pressure differences and penetrability of the main conduits in the phloem.

As was brought out in the previous review (33), the results of Huber and Rouschal's (65) permeability studies were not entirely unequivocal. These workers observed plasmolysis of mature sieve tubes in *Cucurbita* and had difficulty in obtaining uninjured preparations of *Robinia,* that is, preparations in which the sieve-tube contents were not displaced before the plasmolytic agent was applied.

The criticism has been raised that Huber and Rouschal misinterpreted the developmental state of *Cucurbita* sieve tubes, since their illustration of a plasmolysed mature sieve tube showed "an element with a relatively thin uncalloused sieve plate" (22). In reality the plate in the photograph in question is no thinner than is usually found in mature *Cucurbita* sieve tubes (see, for example, Crafts, 21, figs. 2 and 4).

The idea of permeability of sieve elements was soon challenged by Schumacher (105). He tested such elements in 27 different species in many families for their reactions to glucose and sucrose solutions, and reported that all could be plasmolysed at least once, and many could be deplasmolysed one or more times. To be sure that he was dealing with mature cells, Schumacher used, in some plants, elements that were previously found able to conduct fluorescein or elements in fully developed leaves with most of the phloem in differentiated state.

Schumacher (105) stressed the sensitivity of sieve elements to sectioning and cautioned about the exercise of extreme care in the manipulation of material in the study of plasmolysis. The latter usually begins in the median part of the sieve element and may quickly cause a contraction of the cytoplasm into a thin thread. If such a state is reached, deplasmolysis is not readily attained.

The possibility of disruptive action of sectioning upon the sieve-element protoplasts has been suggested in the literature before Schumacher. Crafts (20) replied that phloem cells other than the mature sieve elements were not injured by cutting and therefore the latter could be expected to be no more sensitive. It is well known, however, that the contents of mature sieve elements are readily displaced by cutting, whereas the other kinds of phloem cells are not so affected. The displacement certainly indicates a high sensitivity of mature sieve elements to mechanical manipulations, and Crafts (23) at least agrees that "cutting of sections for microscopic study destroys any conceivable mechanism for rapid phloem transport".

Although Huber and Rouschal (65) originally accepted Crafts' evidence on the permeability of the sieve-element cytoplasm, they were very much concerned about the displacement of contents of these elements in *Robinia* and suggested that the older sieve tubes of this plant might be so penetrable longitudinally ("wegsam") that they cannot be seen in uninjured state in sectioned material. The authors were certain that such sieve tubes were not functionless because phloem exudate could be obtained from them.

Similarly to Schumacher, Curtis and Asai (27) reported that when precautions were " taken to eliminate the disruption resulting from cutting the tissues before making the tests", the semipermeability of sieve-tube cytoplasm could be demonstrated. In the abstract where this report was made, the technical details of the experiment were not described, but according to a personal letter from Dr. O. F. Curtis to the writer the "precautions taken" were as follows: before the tissue was sectioned, its turgor was reduced by causing a severe wilting or by placing the stems, whose epidermis was scraped off, into strong sugar solutions. When such material was sectioned, the sieve-tube protoplasts appeared to have escaped disruption which commonly occurs when turgid phloem is cut. In another method the intact stems were placed into strong sugar solutions, usually about one-molal or stronger, and then plunged into boiling water. Controls were treated with boiling water only. Strong plasmolysis of sieve tubes was observed in the material pretreated with the sugar solution and none in the other.

Curtis and Asai (27) also contended that if the sieve tubes were permeable, their contents, because of high osmotic concentration, would plasmolyse the adjacent perenchyma cells. In support of this contention they stated that they had been able to plasmolyse phloem-parenchyma protoplasts in *Fraxinus* and *Cucurbita* by means of the respective phloem exudates from these two plants. Incidentally plasmolysis of mesophyll cells with phloem exudate taken from the same plants as the mesophyll was observed by Roeckl (91). It is problematical, however, whether this evidence of plasmolysis in tissue severed from the plant can be used to predict the possible situation in an intact plant. As is well known, the effects of cells upon each other are not determined solely by osmotic relationships. Huber (64), for example, assumes that the effect of the osmotic concentration in the sieve elements is more than offset by the turgor pressure in them.

The reference to the absence of cytoplasmic streaming did not remain uncontested either. In a short paper, giving no cytological details, Small (108) reported having seen streaming in *Cucurbita* sieve tubes (interpreted as mature) in a thin strip of tissue connecting two parts of a stem of an intact plant growing in a pot. The strip was exposed for microscopic studies by cutting away the associated tissue. To reduce the operative shock, exceedingly sharp scalpels were used.

In contrast, Rouschal (92), who performed very intensive studies on sieve-element protoplasts, considered the loss of streaming a concomitant of other changes in the maturing cell.

Though occurrence of streaming, if present, would indicate that the sieve-element cytoplasm is rather normal, streaming as such is generally regarded as inadequate to account for the rapid transport observed in the phloem. Even some of the prominent advocates of a diffusional movement in the phloem hold this view (78, 103).

In view of the reports of Schumacher (105) and Curtis and Asai (27), the matter of permeability of sieve-element cytoplasm was obviously in need of further investigation. This task was undertaken by Rouschal in 1941 (92; see also reviews of this work in 63, 64).

This investigator considered first of all the question of sensitivity of sieve elements to sectioning by following the effect of this manipulation upon the phloem in different developmental stages. The familiar displacement of contents of sieve elements began to appear in the sieve tubes of *Robinia* before breakdown of the nucleus, but after the cytoplasm changed its appearance from coarsely granular to very thin and filmy and the sieve-area pores became clearly evident. Peculiarly Rouschal uses the expression "when the sieve pores are broken through". The highly sensitive cytoplasm is characterized as being labile.

In his studies on plasmolysability of sieve elements, Rouschal used two gymnosperms, some dicotyledonous trees and *Cucurbita pepo*. Glycerine, grape sugar, $CaCl_2$, $AlCl_3$, KNO_3, and KCNS were used as plasmolytic agents. In all instances the sensitivity of the sieve elements to sectioning interfered with successful demonstration of plasmolysis. Rouschal then devised a pretreatment, a "preplasmolysis" of phloem in intact state to reduce its turgor before sectioning. The dead bark was removed in a small area, but the functioning phloem was left protected by some living parenchyma cells. Then a paraffin cup containing a glycerine solution was fastened to the exposed area. The cup was cut out on the side turned toward the tree so that the glycerine came in contact with the living cells. In *Cucurbita* the glycerine was injected into the hollow pith area of stems and petioles. This pretreatment did not cause plasmolysis, but made the sieve elements less turgid, and as a result a much greater number of these elements remained intact and could be successfully plasmolysed. Similar results were obtained in the pine by a pretreatment consisting of a hardening of the phloem by $AlCl_3$ and $CaCl_2$ solutions used in place of glycerine. Author mentions also a successful deplasmolysis obtained in his studies.

In considering the details of the plasmolytic reaction of sieve elements, Rouschal stated that during plasmolysis the cytoplasm remained attached to the sieve areas. In other words, the sieve areas constituted the negative areas of plasmolysis.

Although Rouschal was able to induce plasmolysis in mature sieve elements, he continued to regard the latter as highly penetrable, at least in the sieve areas. To test this concept he pretreated with glycerine some phloem of *Aesculus,* prepared sections from it, then introduced some glycerine under the cover glass on one side of the section. Thus a one-sided suction was established and the sieve-tube contents exhibited a rapid longitudinal movement through the vacuoles without any evidence of separation of the cytoplasm from the lateral walls. The flowing sap dislodged the starch grains from their parietal position in the cytoplasm and carried them through the vacuole toward the sieve plate.

According to Rouschal, this experiment demonstrated for the first time, under the microscope, the occurrence of mass flow in living sieve tubes and furnished additional proof of a specific longitudinal penetrability of these elements. He felt justified in concluding that the cytoplasm on the sieve plates was more permeable—at least for water—than the lateral layers.

The glycerine treatment just described failed to cause a longitudinal flow if the sieve plates were plugged (presumably with the proteinaceous material, or the slime; author does not go into the details of this supposed plugging). Rouschal suggests that such plugging is a prerequisite for a successful plasmolysis of sieve elements. If the sieve areas are not plugged, the flow of sap through these areas after sectioning causes the displacement of contents. In natural state, Rouschal contends, the mechanism of plugging may be a means of preventing a loss of materials from sieve elements opened accidentally. Münch's (84, p. 130) observations on the eventual restoration of turgor in phloem that had been incised for obtaining exudate, is supposed to agree with this contention.

Referring to the relative impermeability of the lateral cytoplasm, Rouschal (92) recalled that Münch assumed the existence of such impermeability because he thought the sieve elements occurred next to intercellular spaces. The occurrence of intercellular spaces in the phloem has been reported in the literature, but the phloem has also been described as a compact tissue resembling collenchyma (33). Recently Martens and Pigneur (76) considered this point in some detail by examining phloem of 25 species of dicotyledons and gymnosperms. They used fresh free-hand sections treated with Eau-de-Javelle. Eleven species contained intercellular spaces in the phloem sufficiently close to the cambium that they could have been assumed to be in the functioning part of the tissue; the other 14 did not. Some intercellular spaces occurred next to sieve elements.

Rouschal (92) reinvestigated the reaction of the phloem to neutral-red treatment. He stained the tissue in the tree and in severed sections. In both conditions differentiated sieve elements did not accumulate neutral red, and this property appeared even before the cells were fully mature. The same sieve elements which failed to accumulate the dye could be plasmolysed. Using fluorescence microscopy, Rouschal checked the possibility that the sieve elements, because of their alkaline sap, might accumulate the base of the dye rather than the dye itself. No such base accumulation was detected in mature sieve elements. The young elements and the cambium contained both the dye and the base of the dye.

Rouschal assumed that the failure of mature sieve elements to accumulate neutral red resulted from a combination of alkaline state and scarcity of fatty substances and tannins. His own tests for lipoidal substances with OsO_4 gave a slight darkening of cytoplasm and no color reaction in the vacuole. Preliminary tests with rhodamin B also gave negative results regarding presence of lipoids. Among the preceding workers, Schmidt (98), too, found no accumulation of fatty materials in the sieve tubes. As mentioned earlier, Salmon (95) claimed to have found a progressive increase of lipoids in the slime of differentiating sieve tubes.

That tannins play a role in the accumulation of vital dyes in certain type of vacuole is well known (130). Lipoids, on the other hand, are usually associated with the cytoplasm rather than the vacuole. However, Bailey and Zirkle (6, 130) think that fatty substances may well exist in the vacuome, and Scarth (96) has shown that in certain vacuoles the colloidal contents act as partially hydrated lipoids. Regarding the effect of pH, Zirkle (130, p. 9) states that neutral red " does not collect in vacuoles more basic than pH_6".

Although the behavior of the fatty materials in the differentiating sieve-element protoplast needs further study, observations made thus far indicate, according to Rouschal, that during maturation, fatty acids and neutral lipoids diminish in amount and the protoplast and cell sap become more and more hydrophilous. The alkalinity of the sap may also favor hydration, especially since K alkalies prevail. Rouschal finally concludes that the hydration of cytoplasm is responsible for its labile state, its low viscosity and highly fluid condition, and its appearance as though it were optically empty when examined with surface illumination. Huber and Rouschal (65) and later Rouschal (92) attempted to study sieve elements in their natural position in a plant by fastening a dark field microscope to a tree. The contents of parenchyma cells were discernible, but the sieve elements appeared optically empty.

Crafts' (26) idea of a viscous, tough, fibrous condition of the sieve-element cytoplasm obviously conflicts with Rouschal's (92) picture of a highly fluid fine ("zart") cytoplasm, thin as breath ("hauchdünn"); but the thinking of the two investigators converges on the concept of a highly hydrated state of this cytoplasm.

According to Rouschal (92), one of the most important unexplained matters regarding the cytophysiologic characteristics of the sieve element is that of the relation between the vacuole and cytoplasm; in other words, the matter pertaining to the nature of the tonoplast. He was unable to detect a tonoplast in any experiments. In connection with this problem, the cause of the high permeability of the cytoplasm at the sieve areas also cannot be fully explained at present. Moreover, this permeability has been experimentally shown to exist for water and fluorescein alone, whereas passage of sugar and protein precursors can be only assumed from the physiologic evidence of movement of materials through the phloem.

In contrast to Rouschal, a student of Schumacher, A. Schumacher (101) reported having found evidence of the existence of a tonoplast. Similarly to Schmidt (98), she described cytoplasmic strands crossing the vacuoles. However, author found these structures in relatively few sieve tubes and did not mention the stage of development of these elements.

While the plasmolytic studies of W. Schumacher and Rouschal contradict the idea of passivity of the sieve-element protoplast, the investigations of Roeckl (91), a student of Huber, supply new evidence that the movement from the mesophyll toward the phloem does not follow a gradient from a relatively high to a relatively low pressure. In a detailed study of the osmotic relations in the mesophyll and phloem, Roeckl found a gradually but noticeably increasing osmotic pressure from the palisade parenchyma, through the collecting cells (50, p. 126) of the spongy parenchyma and the border parenchyma of the bundles, toward the phloem. Thus the movement from the place of elaboration of carbohydrates to the sieve elements seems to occur against the concentration gradient.

THE SHAPE OF SIEVE ELEMENTS, AND ITS RELATION TO FUNCTION. In Craft's (22) opinion the shape of the sieve elements and the structure of the sieve plate are of little consequence with regard to translocation; these elements are assumed to have cytoplasm as permeable as the walls, permitting therefore ready passage of inorganic nutrients and organic assimilates, regardless of the presence of sieve plates. Recently he (26) developed this concept further by suggesting that, like the cytoplasm, the sieve-element walls too may be highly hydrated and permeable, and therefore specialized for conduction.

The German phloem investigators did not adopt Crafts' view on the unimportance of sieve plate in translocation and continued to search for the significance of size and distribution of the sieve areas in the sieve elements, of the pores in the sieve areas, and of the variations in these characteristics. Huber (62) was concerned about the observed trend in the evolution of the sieve elements from long-spindle-shaped cells to short elements with more or less transverse end walls; in other words, a trend toward an increase in the number of sieve plates to a unit of length of the phloem strand. He concluded, however, that no actual increase in resistance to flow resulted from this evolutionary sequence because of the concomitant widening of the elements and particularly because of a change from many small to few large pores in the sieve plates. This trend, according to Huber, answers the demands of the law of Poiseuille for mass movement. Münch (85), concurring with Huber, made detailed calculations of possible rates of movement, considering the width of the sieve elements, the number, diameter and length (corresponding to thickness of sieve-area wall) of pores, and concluded that in the angiosperms and gymnosperms the area covered by the pores is approximately as large as the transection of the sieve element.

In contrast, Schumacher (101) found in *Bryonia dioica* that the sieve-plate pores, including the callus cylinders, occupied only 46 to 68 per cent of the sieve-plate area. If measured within the callus cylinders, the pore area is reduced to from four to six per cent of the sieve-plate area. The last values closely approach those previously obtained by Crafts (19) for potato sieve tubes; in these the strands were reported to occupy 2.3 per cent of the sieve-plate area. Schumacher (101) emphasized that while the sieve-tube lumen was reduced within the pores, the internal surface, that is, surface covered with cytoplasm, was increased two to four times.

Cheadle and Whitford (15) suggested that the orientation of the sieve plates has some bearing on the efficiency of sieve tubes in conduction. Since they found sieve-tube elements with transverse end walls and simple sieve plates in leaves, in annual and storage organs, and in plant groups which seem to have highly specialized vascular elements in general, they advanced the notion that such sieve-tube elements are highly specialized with regard to food conduction.

The proper conclusion regarding the role of the shape of the sieve element in the performance of its function will not be drawn, of course, until this function is properly understood.

THE NATURE OF CONNECTING STRANDS. The question whether the connecting strands in the sieve areas are purely cytoplasmic or whether the vacuolar contents are also connected through the pores has played a prominent part in the speculations about the function of the sieve-element. To interpret the connecting strands as cytoplasmic and to refer to them as "plasmodesmata" has become rather common in modern phloem literature (22, 33, 66, 78, 92). In contrast, some of the old workers assumed continuity of vacuolar contents through the pores in uninjured sieve elements.

According to the concept of Crafts (22, 26), the absence of vacuolar connections through the pores in the sieve areas would not prevent free flow across the plate because the cytoplasm and the wall component of the sieve area are both highly permeable.

Without referring to Crafts, Rouschal (92) expressed a somewhat similar idea when he stated that the submicroscopic pores of the cell walls would suffice for movement of water and sugar across the sieve areas, especially in view of the evidence of high cytoplasmic penetrability in these areas. Nevertheless, he also speculated on the possible structure of the connecting strands that may make them permeable: perhaps these strands were composed not of semi-permeable membrane alone but of inner cytoplasm delimited by a membrane along the pore walls. Thus at least one semi-permeable barrier would be non-existent. Absence of a tonoplast would further increase permeability.

The sieve-element slime is commonly present in the sieve-plate pores in sectioned material. Such distribution of this matter has been interpreted as an artifact in most of the modern and some of the old literature (see 33). Recently, however, Schneider (100) thought he saw evidence of normal continuity of slime between elements. In *Vitis* used by the present writer (44) occasional dormant sieve tubes had dense masses of slime stretched through the whole length of the elements and connected by single thick strands through the sieve plates. The latter were covered with thick dormancy callus at this time, except where the strands occurred. These views did not suggest a response "to intense and sudden steepening of the pressure gradient by cutting" (21), since the sieve tubes were in dormant state and the slime did not occur as "slime plugs" on the sieve plates. Possible normal association of the slime with the sieve plate perforations in *Vitis* is also indicated by the reappearance of slime strands in the callus during reactivation of the sieve tubes. These strands become evident before the dormancy callus is removed, and at first they penetrate the callus mass only part way (44, plate 20, *A*), sometimes from both sides at the same time.

Another significant observation is that the characteristic chromaticity of the connecting strands in the differentiating sieve elements and the firm attachment of the protoplast to the sieve plate become evident approximately at the time when the nucleus and slime bodies disintegrate (32, 44). Moreover, only the connecting strands show the increased chromaticity, whereas the cytoplasm along the lateral walls remains lightly stained. If the dense staining of the connecting strands occurs because the cytoplasm composing them shows affinity for dyes (26), then one should assume that this cytoplasm differs from that in the parietal layers which lose their chromaticity during maturation (26). An alternative possibility would be that the slime is in some way associated with the cytoplasm of the connecting strands and makes them chromatic.

Occasionally the literature reports that the connecting strands in the sieve areas are not continuous. Mangenot (75), for example, considered both the pit connections in red algae and the sieve-plate connections in dicotyledons as discontinuous structures. According to many workers (see 51, p. 447), the pits in the Florideae have pit-closing membranes with fine plasmodesmata connecting the masses of cytoplasm that fill the pit cavities. Mangenot calls these masses "plasmodesmata", denies the presence of pit membranes and suggests that the "plasmodesmata" are individually invested in their own plasma membranes. From a brief study of sieve tubes of some angiosperms he drew a similar conclusion about the connecting strands in the sieve plates; in some plants he saw a light line in the median part of each connecting strand and interpreted this line as a discontinuity. Salmon (95) followed Mangenot in describing the connecting strands in the sieve plates of angiosperms as being double, though she too found the "light line" dividing the strands in halves an inconsistent characteristic.

It is of interest to recall that the earliest workers on phloem macerated this tissue and pictured very much extended but uninterrupted connecting strands of sieve plates in which only the intercellular substance or all wall substances were removed (57, 59, 125; see also 33, fig. 3).

SIZE OF THE SIEVE-TUBE SYSTEM AS A WHOLE. A unique attempt to relate the dimensions of the entire sieve-tube system of a plant to the activity of this system, as expressed in the conduction of fluorescein, was made by A. Schumacher (101), a student of W. Schumacher. The number of sieve tubes, their transsectional areas and the length of their elements were determined at different levels of several herbaceous and woody plants. The values for the transsectional area and length of elements served to calculate the volume and the internal surface (surface of the lateral walls and of the two sieve plates) of the sieve elements.

One of the staminate plants of *Bryonia dioica* which was studied in particular detail gave the following values. The main axis of the plant was 7.4 m. long and bore 73 leaves, 73 tendrils, 28 inflorescences and five lateral branches. The latter taken together were 13.8 m. long and bore 140 leaves, 140 tendrils and 44 inflorescences. The counts and measurements were made a few cm. apart in all organs of the plant including the roots. The calculated totals indicated the presence of approximately 80 million sieve-tube elements in the entire plant. These had an internal surface of one sq. m. and a total volume of only four cubic cm.

In the main axis 300 to 400 sieve tubes were found in one transsection. In one centimeter of stem these sieve tubees occupied a volume of two to three cubic millimeters and had an internal surface of 300 to 400 square millimeters. This surface exceeded the outer stem surface three to four times.

In the longitudinal course through the main stem and the tap root, including the enlarged part of the latter, the volume and the internal surface of the sieve tubes decreased toward the apices. The number of the sieve tubes diminished only slightly in the stem, very strongly in the root. In the lateral organs the conducting elements were of smaller dimensions than those in the main axis and were reduced in volume and internal surface toward the apices.

The volume of the sieve tubes in the main axis was about equal to that of the entire lateral system, but the latter had a three times larger internal surface in all its sieve tubes. Thus the downwardly moving materials would encounter no reduction in relative volume, but a decrease of internal surface in the part of the conducting system where the lateral branches join the main stem.

Author also studied the movement of fluorescein in various parts of *Bryonia dioica* plants, related this movement to the size relationships in the sieve-tube system and suggested that the cytoplasm proper may play an important role in the transport of assimilates.

CONCLUSIONS. The data reviewed show that the opinions regarding the characteristics of the protoplast of a sieve element continue to be divided. Most evidence suggests that the mature functioning sieve element has no nucleus; that its vacuole is not clearly defined; that it is very sensitive to mechanical manipulations and responds to these with a marked displacement of its contents; that when special precautions are taken to preserve the protoplast in its natural state, it can be plasmolysed and even deplasmolysed, but cannot be made to accumulate vital stains; and that there is at least a cytoplasmic continuity from element to element.

Although it is commonly accepted that materials move rapidly through the sieve elements, the views of the proponents of the mass flow and of those of a diffusional movement are not reconciled about the particular characteristic which determines the rapid movement. There is experimental evidence, however, that materials pass readily across the sieve areas.

PHLOEM DEVELOPMENT

PRIMARY PHLOEM. The basic features of the development of primary phloem and the concepts of protophloem and metaphloem have been discussed in detail in two reviews by the writer (33, 38). Since then several new articles dealt with vascular initiation and have furnished data which are in general agreement with the conclusions drawn previously.

Firstly, in a growing shoot or root the initial sieve elements mature closer to the apices than the initial tracheary elements; in other words, within the procambium the first sieve elements appear earlier than the first tracheary elements at a given level of stem or root. Such timing in vascular differentiation has been recorded in the shoots of gymnosperms (24, 25, 56, 110, 111), dicotyledons (31, 35, 41, 44, 82, 89, 100) and monocotyledons (40, 106), and in the roots of various plants (39, 126). Not all the references just cited give an accurate description of the first sieve elements. Usually, however, the latter can be identified as such in the accompanying illustrations by the similarity in position and appearance with elements that have been definitely recognized as sieve elements by some other writers. Papers presenting contrasting views, that xylem elements differentiate before (10) or simultaneously with (17) the first phloem elements, give no satisfactory evidence that the initial sieve elements were actually seen by the authors.

Secondly, in gymnosperms and dicotyledons the first sieve elements show a usually continuous acropetal differentiation within the shoot axis and from the axis into the leaves (24, 25, 31, 36, 41, 56, 82, 89, 110, 111)[5]. In their acropetal differentiation the sieve tubes may show discontinuous maturation. Thus in both flax and sunflower studied by the present writer (36, 41) some leaf traces showed series of immature sieve-tube elements interpolated between series of mature ones.

The sequence of protophloem, metaphloem and secondary-phloem development was investigated in detail by Schneider (100) in the peach and by Esau (44) in the grapevine. In both plants fibers differentiate in the protophloem after the sieve tubes are obliterated. The metaphloem has no fibers. This pattern of differentiation is in keeping with that established for many other dicotyledonous plants (33). Schneider (100) points out that in the leaf blades and petioles of peach the protophloem cells other than the sieve-tube elements elongate much but do not complete their development into fibers; they remain without secondary walls. This difference in the characteristic of the old protophloem in leaves and stems is common in dicotyledons and is well illustrated by *Nicotiana* (32), *Cannabis* and *Corchorus* (69).

Schneider (100) has found, in the peach, a lag between the initiation of the last metaphloem cells and the start of production of secondary phloem. Moreover, the last cells initiated on the phloem side by the procambium matured as large parenchyma cells, and thus the metaphloem became sharply delimited from the secondary, at least in the internodes. Whether the two kinds of tissue are interconnected in the nodes has not been investigated.

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[5] According to recent studies (64*a*), the protophloem and metaphloem in the internodes of *Equisetum* differentiate basipetally. This course of differentiation appears to be related to the protracted intercalary growth at the base of the internodes. It would be of interest to determine the course of phloem differentiation in monocotyledons having a similarly prolonged intercalary growth of internodes as *Equisetum*.

SECONDARY PHLOEM. The seasonal development of the secondary phloem has received some attention in the new literature. The research previously reviewed by the writer (33, pp. 419–421) and certain other references (67, 87, 100) suggest that in most angiosperms the secondary phloem functions only one season, but some cambial derivatives on the phloem side pass the winter in undifferentiated state and complete their maturation in the spring before the cambium resumes its activity. Huber (62) reexamined the seasonal changes in several dicotyledonous and gymnospermous trees. By using mainly transverse sections from material preserved in alcohol, he determined how much of the phloem contained collapsed sieve elements at different times of the year. Callus development and changes in contents of the cells were not followed in detail. According to Huber (62), in the dicotyledons either all sieve tubes are collapsed before the cambium is reactivated or a few of the last-formed narrow sieve tubes are still open. This observation agrees with that of Gill (53) who also found, in *Fraxinus excelsior* and *Quercus robur,* some narrow-celled autumn phloem which was not crushed until the second season of its existence.

In certain Abietineae Huber (62) observed that a considerable part of a season's growth remained intact through the winter and did not collapse until late in the second season. Thus in a given growth season the active phloem band was eventually made up of two parts formed during two successive seasons. In *Picea excelsa* the latest row of cells of a given season collapsed in the spring and could be used as a marker between the two different parts.

Apparently the sequence in the seasonal development of the Abietineae phloem, reported by Huber (62), is not generally known. Raatz (88), however, seems to have referred to the same phenomenon in 1892 when he mentioned the persistence of sieve cells in *Picea excelsa* and the collapse of the latest row in a given season's increment before the beginning of new growth. Brown's

(13) description of phloem development in *Pinus strobus* implies that part of a season's growth does not collapse in the fall and survives through the winter. He calls this tissue " transitional cells". According to Abbe and Crafts (1), in *Pinus strobus* some sieve elements remain immature during the winter and then complete their development in the spring.

As a side issue of his studies on the plasmolysability of sieve elements, Rouschal (92) noticed that in trees this property changed strikingly during the course of one summer. Plasmolysis was easy to demonstrate till the end of July. After this time the protoplasts became very sensitive to sectioning. This change occurred in the phloem that was formed during the season when the tests were made and, in *Pinus,* also in those elements that were carried over in a non-collapsed state from the season before. The increase in sensitivity of the sieve-element protoplasts appeared to coincide with the beginning of the state when exudate could be obtained from the phloem. Huber (64) finds no explanation for this late start of exudation in trees, but sees in it an indication of a lack of exact parallelism between movement of assimilates and occurrence of exudation.

According to the literature just reviewed, the secondary sieve tubes of dicotyledons are commonly short lived and become functionless during the same season when they are initiated in the cambium. Because of the relative longevity of their sieve elements, *Tilia* and *Vitis* are the recognized exceptions among the dicotyledons (33, 62). In *Tilia* the sieve tubes are reported as remaining active for several years without development of callus during the winter. *Vitis* sieve tubes, on the contrary, deposit large masses of transitory (or dormancy) callus as the plant becomes dormant and remove this callus again the following spring (44, 125). In other words, *Vitis* phloem shows an alternation of dormancy and reactivation, and these phenomena may occur more than once in the same sieve tubes (44). Usually, however, the sieve tubes of *Vitis* cease to function during the second summer after they are formed, and the inactive phloem is cut off by cork. Definitive callus may or may not develop before the elements cease to function.

Apparently, besides *Vitis,* only *Tecoma* and certain monocotyledons have been observed forming transitional callus during the winter dormancy (see literature in 33, 44.) Although

Bliesenick (9) spoke of resumption of activity in the spring by the old sieve elements, as though it were a common behavior in many plants, he failed to distinguish between transitory and definitive callus—at least, he did not mention the latter in describing phloem obliteration. His reports on reactivation require, therefore, a reexamination.

FUNCTIONLESS PHLOEM. The terms "nonfunctioning" or "functionless phloem" are commonly used to designate a tissue that ceases to be concerned with the primary function of the phloem, viz., conduction of organic solutes (33, 44). The sieve elements and companion cells (or their analogues) are dead and more or less collapsed in such phloem, but the other parenchymatous elements or other types of living cells (e.g., septate fibers, 44) continue to take part in the metabolism of the plant. This is evidenced by starch storage in them. Thus the difference between functioning and nonfunctioning phloem is determined by the condition of the sieve elements and the cells specifically associated with them.

The characteristics of non-functioning phloem vary in different plants. The appearance of the primary phloem after it ceases to conduct has been considered in the previous review (33) and also in the parts dealing with phloem fibers and primary phloem in the present paper. Commonly the primary sieve elements (and companion cells, if present) are more or less crushed or entirely obliterated by pressure from the surrounding cells. Crushing of sieve elements occurs in the secondary phloem also. If the latter contains many fibers, the sieve elements may remain open, but devoid of their original contents (e.g., 44, 52, 100).

Vitis appears to be the only plant recorded in the literature as having tyloses in old sieve tubes (44, and the literature reviewed there.) These tyloses arise from phloem-parenchyma cells in the part of the functionless phloem that fails to be cut off by the cork cambium. They store starch and sometimes develop thick walls.

Commonly definitive-callus formation accompanies the loss of function by the sieve elements, whether this loss occurs normally or under the influence of diseases or other injurious agents (43, 102). If the functionless phloem is not quickly sloughed off, the definitive callus may be again removed. How this removal occurs in the presence of a disorganized protoplast is not clear.

The death of companion cells in angiosperms and of albuminous cells in gymnosperms has been emphasized in the previous review (33).

In contrast to all other reports on inactive sieve elements, Shimakura (107) contended that in the rhytidome of conifers the sieve cells, as well as the phloem parenchyma, underwent expansion. Normal sieve cells were neither pictured nor described by this writer, and the identity of these cells in his illustrations of the rhytidome is very uncertain. The expansion of phloem-parenchyma cells in the non-functioning phloem is a familiar phenomenon. It appears to be the primary cause of crushing or obliteration of sieve elements (33). The latter, however, as enucleate cells are not known to be capable of further growth after the loss of nucleus.

PHLOEM DEVELOPMENT IN RELATION TO FLOWERING. Wilton, Struckmeyer and Roberts reported in a series of papers (112, 113, 128, 129) that the essential anatomic characteristic of the flowering stems of herbaceous plants is the slowing down or, if reproductiveness is strongly expressed, a complete cessation of cambial growth. As a result of this interruption of meristematic activity, xylem and phloem mature to the cambial initial zone. The phloem of reproductive axes shows fewer and smaller cells than those of the vegetative. In general this tissue appears in flowering axes quite mature and even senile because of the thick walls, the pronounced callousing of sieve plates and the marked crushing of sieve tubes (90, 113). The cessation of cambial activity and the concomitant phenomena spread downwards in flowering axes (128). A renewal of vegetative growth is accompanied by a resumption of cambial activity (113, 114, 129). The anatomic modification characteristic of flowering plants can be quickly induced by placing plants in environment favorable for flowering, and the changes occur before flower primordia become microscopically discernible (112).

By comparing the anatomy of leaves in topped and untopped tobacco plants, Avery (5) found that vegetative growth ceased soon after the seeds started to form. Topping stimulated cambial activity which, however, resulted in xylem production only. Phloem cells, on the other hand, developed in greater numbers in untopped plants forming seeds. Thus Avery's data regarding phloem differentiation in fruiting and nonfruiting plants seem to differ somewhat from those of Wilton, Roberts and Struckmeyer (112, 113, 128, 129). Whether this difference is a result of the use of different kind of material in the two sets of studies (Avery using leaves, the others stems) is not obvious at present.

The data on phloem development in relation to flowering in woody species are incomplete. In the alternately bearing apple, the spurs that initiate flower primordia on a non-bearing tree ("off-year" spurs) show the flowering type of anatomic structure four to five weeks before the primordia are apparent (115). Although cambial activity ceases early in such spurs, Finch's (47) observations indicate that the initial radial growth proceeds in them so vigorously that they have in the end a much wider increment of xylem than spurs not forming flower primordia. Whether the phloem too is wider in the spurs that are preparing for fruit-bearing is not indicated. Even if this phloem were wider, it could become functionless at the end of the season. Then, presumably, cambial activity in the same spur would form new phloem at the beginning of the new season, the season when the flowers would expand. Apparently this particular matter has not been investigated in the apple. In an early paper Finch (46) reported that cambial activity in "one and two year old wood" of non-bearing trees started later but proceeded more rapidly than in similar "wood" of bearing trees. He did not specify, however, whether this "wood" was part of the fruiting spurs in the bearing trees.

Gill (54) found considerable differences in the relation between flowering and cambial activity in different amentiferous tree species. *Corylus avellana* and *Alnus glutinosa* have fully differentiated catkins at the beginning of the winter, and their expansion in the spring is associated with no renewal of cambial activity in the inflorescence axis. In contrast to these two species, *Populus serotina* and *Salix caprea* have, during the winter, rather undifferentiated catkins, and when the latter expand in the spring renewed xylem and phloem differentiation occurs in their axes; furthermore, some phloem (but no xylem) differentiates also throughout the twigs below the expanding catkins.

The physiologic implication of these various observations has not yet been properly interpreted. The discontinuance of cambial growth in flowering stems of herbaceous plants, as emphasized by

Wilton, Struckmeyer and Roberts, suggest that maturation of vascular tissues is a concomitant of the initiation of the reproductive state, whereas the vegetative condition is associated with continued formation of vascular elements. In the woody species, too, a cessation of cambial activity and of growth in length occurs in the preparation for flowering. But flower primordia are initiated in one season and fruits develop in the second. The behavior of the cambium of a fruit-bearing spur (the cambium which early ceased its activity during the previous season) and the condition of the phloem of such a spur is apparently not known.

PHLOEM FIBERS

PRIMARY-PHLOEM FIBERS. Considerable information has been gathered during the last ten years proving that the fibers occurring on the periphery of the vascular system in many dicotyledonous stems (the "pericyclic" fibers) are primary phloem fibers (see 37, 44, and the literature reviewed there.) In other words, these fibers arise from the same part of the procambium as the earliest sieve tubes, and the latter usually occur among the cells that subsequently mature into fibers. During elongation of the organ in which they differentiate, the first sieve tubes are more or less obliterated and the fibers mature by ceasing to elongate and by developing secondary walls. This sequence of development has been observed during the last few years in *Cannabis* and *Corchorus* (52, 68), *Linum* (37), *Prunus* (100), *Cryptostegia* (4) and *Vitis* (44). The phloic[6] origin of the peripheral fibers has been previously discussed by the present writer in relation to the question whether or not these fibers should be termed "pericyclic" (33, 37, 38, 44).

If the primary-phloem fibers are considered in the light of the concepts of "protophloem" and "metaphloem" (38), they appear to fit best into the category of "protophloem fibers" because they and the sieve tubes associated with them are initiated before the elongation of organs in which they occur. The subsequent phloem, the metaphloem, whose sieve tubes mature after the organs elongate and persist longer than the protophloem sieve tubes, is usually free of fibers, especially if fibers occur in the protophloem (33).

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[6] This term is used as proposed by Esau (44): "The word 'phloic' is adopted here as an adjective derived from the word 'phloem'. A precedent in the use of the adjectival form of 'phloem' occurs in the well known terms 'ectophloic' and 'amphiphloic'".

The differentiation of most or of all cells that remain intact in the protophloem after obliteration of the sieve tubes raises a provocative question regarding the possible functional relation between the future fibers and the protophloem sieve tubes while the latter are still intact. These sieve tubes, especially the earliest among them, may be without companion cells (33). Students of phloem usually think of the function of sieve tubes as being at least partially controlled by the normal nucleate cells associated with them. Perhaps the future fibers, which appear to be the only nucleate cells in the protophloem of some plants, play a similar role in the function of this tissue as phloem parenchyma or even companion cells.

The cells acquiring secondary, often lignified walls in the old primary phloem sometimes do not develop into typical fibers; they remain relatively short and their ends do not become much pointed. It seems entirely proper to regard these cells as sclerified phloem parenchyma. Wilson (127) treated thus the lignified cells of the primary phloem of the many Compositae which he studied. Russow (94) considered that the thick-walled lignified cells in vascular bundles of some Compositae, Gramineae, Liliaceae and Alismaceae are not "Bastzellen" but are derived from the "Weichbast"; that is, they are simply the modified parenchymatous elements of the phloem.

Whether there is any fundamental difference in the physiologic relation between the sieve tubes and young fibers on the one hand and between the sieve tubes and phloem parenchyma (which may or may not become sclerified) on the other, is a question that cannot be answered. At present one can only record the existence of variable development of the sclerenchyma during primary vascular differentiation. In some plants the strengthening cells are initiated as such very early and eventually develop the characteristics of typical phloem fibers (e.g., *Linum, Cannabis, Cryptostegia*); in others the supporting tissue differentiates "secondarily" from phloem parenchyma (e.g., certain Compositae); in still others sclerenchyma arises not in direct association with the vascular tissues but outside of them (e.g., *Cucurbita, Aristolochia, Tradescantia*).

SECONDARY-PHLOEM FIBERS. In the previous review on phloem (33) a positive statement appears on page 412 that "the ontogenetic relation between the fibers and the other members of the secondary phloem is clearly understood. When present, the fibers arise, together with the other elements of the longitudinal system, from fusiform initials of the cambium". Since these lines were written, a different, although not a new, aspect of fiber development in the secondary phloem has attracted reviewer's attention. In some species the secondary phloem shows no fully differentiated fibers while the sieve tubes are in functioning state. Only after the latter become senile, fibers mature. In the cherry (100) and guayule (3), for example, sclerification of the secondary fibers is delayed until the sieve tubes cease to function. In guayule it seems impossible to distinguish between the phloem-parenchyma cells and the "sclerenchyma initials, since most of these cells are pointed and eventually thicken and lignify" (3). Perhaps fibers, while not fully mature, sometimes replace phloem parenchyma in the secondary phloem as they appear to do in the primary.

Generally developmental relations suggest the following three categories of fibers in the secondary phloem: (*a*) fibers maturing at the same time as the sieve elements and appearing to be highly specialized as mechanical elements (e.g., *Tilia*); (*b*) fibers maturing at the same time as the sieve elements but retaining active protoplasts that are concerned at least with the carbohydrate metabolism (e.g., starch-storing septate fibers of *Vitis*); (*c*) fibers maturing after the sieve elements cease to function and sometimes resembling the phloem parenchyma cells in the functioning part of the phloem (e.g., cherry, guayule).

Thus the statement made ten years ago and cited in the beginning of this section of review must be revised as follows: " The ontogenetic relation between the fibers and the other members of the secondary phloem is not always clearly understood, since in some plants the fibers mature late and cannot be distinguished from phloem parenchyma in the functioning part of the phloem". The commonness of the loss of protoplasts by fibers (33, p. 413) may be questioned also in view of Kundu's (68, 69) report that even such typical phloem fibers as those of *Cannabis* and *Corchorus* remain alive throughout the life of the plant.

PRIMARY-PHLOEM FIBERS AND THE PERICYCLE. As was previously implied in this review, identification of primary-phloem fibers as such has a bearing upon the question of usage of the term "pericycle". The present reviewer has repeatedly advocated abandonment of this term with reference to the peripheral region of the cauline and foliar vascular system of Spermatophyta in which this region is obviously a part of the phloem. No separate region intervenes between the phloem and cortex in such stems, and the leaves associated with them have no pericycle either. The roots, however, usually show a layer or more of tissue outside the vascular elements, a region that may continue to bear the name of "pericycle", at least for the present.

One might object that retention of this term for roots and its abandonment with reference to shoots will destroy the idea of unity of shoot and root. However, the lack of absolute homology of morphologic regions in stem and root is generally recognized. For example, according to Schneider's Handwörterbuch der Botanik (99), the pericycle of root and stem are non-homologous, ontogenetically and physiologically, and therefore the region between the endodermis and the vascular tissues should have different names in the two organs—"pericycle" in the stem, "pericambium" in the root. Recently Allen (2) concluded that, on the basis of histogenesis in embryos of *Pseudotsuga,* "the primary root is homologous with the whole primary shoot having in addition an outer mantle of tissue which has no counterpart in the shoot" and no region exists in "the primary root which can be considered homologous with the cortex of the shoot unless the root pericycle is cortical in nature".

It was stressed in one of the previous reviews (38) that Van Tieghem, in introducing the concept of "pericycle", disregarded the ontogenetic aspect of the problem. Later, however, in his study of the cryptogams (117, 118, 120) he paid attention to ontogeny and decided that *Equisetum* and certain ferns have a double endodermis and no pericycle; that in *Polypodium vulgare* the large steles have a pericycle, the small one has none. Van Tieghem (119) also contended that leaves and astelic stems have no pericycle, because if a stele is absent, so is the "conjunctif", and without the latter there are no pith, no rays and no pericycle. He proposed the term "peridesme" for the parenchyma between the endodermis and the vascular tissues in astelic organs. In an early paper written before the introduction of the term "pericycle", Van Tieghem (116) reported that, whereas the "rhizogenous membrane" (pericycle) occurred between the vascular tissues and the "protective layer" (endodermis) in the root of a seedling, at higher levels of the latter, where the stem structure appeared, the phloem cells were touching the endodermis. Dangeard (28) and Fischer (49) made similar observations. These examples of Van Tieghem's treatment of the "pericycle" show that even the creator of the term allowed for a certain variability in the fundamental plan of plant structure and could envisage the absence of pericycle.

It is useful to recall also the opinion of Brebner (12)—the investigator whose name is so prominent in the literature dealing with the concept of stele—on the morphologic value of pericycle. He appreciated the advance made by Van Tieghem "in emphasizing the importance of the vascular conducting cylinder as a whole by his stelar terminology" but also felt "that a certain number of the terms did not fit" the views resulting from newer studies. For example, Brebner thought that the histogenetic layers, the endodermis and the pericycle "should be, in many cases, abandoned as morphologic criteria" and that perhaps the phloem should be fixed as the outermost limit of the stele because the "pericycle" is so inconstant in its origin. In fact, according to Brebner (12, p. 548), "it would perhaps have been better to abandon the stelar theory altogether, and substitute for it the conception of a connected system of vascular strands on the one hand and nonvascular on the other. At the same time the nonvascular tissue would not be considered as something essentially and fundamentally different from the vascular for they both are differentiated from the same meristem, and what has become vascular in one case might very well be nonvascular in another and *vice versa*". And finally (12, p. 549), "perhaps the time is hardly yet ripe, but the day does not seem far off when the three histogenetic layers, as well as cortex, pith, endodermis, pericycle, etc., will have lost to a great extent their present morphological connotation and be used simply as convenient descriptive terms in certain cases". Since Brebner wrote this significant statement, the "three histogenetic layers" have lost their morphological connotation, and the time has become ripe to reexamine thoroughly the concept of "pericycle"; and it seems obvious that the origin of the term "pericycle" and the treatment and critique of it by investigators prominently involved in the evolution of the stelar concept do not support the recently expressed contention (8) that "the background for the term 'pericycle' is much broader than the present bases for negating its existence."

In the delimitation of tissue regions in plant organs, physiologic considerations are perhaps as useful as the ontogenetic. Van Fleet (121–123) has clearly shown, for example, that the characteristics of the limiting layer between the vascular region and the cortex are determined by the reaction of substances originating in the two regions. Thus, although the endodermis of the root may not be homologous with the innermost cortical layer of the stem, physiologically both are analogous; both represent "a boundary between two physiologic systems, the cortex and the stele", where the "effusa from one system may become halted and deposited at the boundary of another" (121).

In the "endodermis" as interpreted by Van Fleet we have a practical topographic as well as functional concept. It enhances our understanding of the inter-relation between tissue regions and gives a concrete basis for regarding the plant body as an organized unit. In contrast, introduction of the term "pericycle" in the purely morphologic sense of Van Tieghem has for many years obscured the understanding of structure and differentiation of the primary phloem and has perpetuated an erroneous idea about the nature of the peripheral part of the vascular system in the higher plants.

To conclude the consideration of the pericycle, a list of families is appended whose representatives (one or more) were found to have the primary phloem in contact with the cortex. Léger (72) contributed the largest number of names to this list: Apocynaceae, Asclepiadaceae, Boraginaceae, Chenopodiaceae, Compositae (Radiatae, Tubuliflorae, Liguliflorae), Cruciferae, Hippocastanaceae, Labiatae, Leguminosae, Lythraceae, Magnoliaceae, Malvaceae, Menispermaceae, Onagraceae, Polygonaceae, Rosaceae, Scrophulariaceae, Solanaceae. To these may be added Linaceae (37), Moraceae (68), Tiliaceae (68) and Vitaceae (44). In the following families Léger (72) was not prepared to deny the presence of a pericycle because he found a separate tissue region intervening between the phloem and cortex: Caryophyllaceae, Commelinaceae, Cucurbitaceae, Geraniaceae, Liliaceae.

CONCLUSION

The present review may be appropriately terminated with a similar summation of the concept of phloem as the previous review (33) but revised with a view on the investigations of the last decade.

The modern concept of phloem is that of a tissue containing highly specialized cells, the "sieve elements", which together with the parenchymatous members of the phloem form anastomosing strands as a part of the vascular system. The protoplasts of the sieve elements are intimately connected with each other through localized wall portions occupied by "sieve areas" which are comparable to pits in their origin and fundamental structure. The connecting strands in the sieve areas vary greatly in size but are usually more conspicuous than plasmodesmata and are typically associated with variable amounts of callose in the wall region they traverse. The slime which occurs in larger or smaller amounts in sieve-element protoplasts appears to be associated in some obscure manner with the cytoplasm of the connecting strands. In the lower vascular plants and the gymnosperms the sieve elements usually have one type of sieve area on various walls and are called "sieve cells". In angiosperms the sieve areas of certain walls, usually those of the end walls, are particularly well differentiated and form "sieve plates", simple (one sieve area) or compound (two or more sieve areas). The sieve elements of the angiosperms, the "sieve-tube elements", are arranged in longitudinal rows and form thus the "sieve tubes". The protoplasts of the sieve elements lose their nuclei upon maturation and become highly sensitive to mechanical manipulations to which they commonly respond with displacement of contents. The parietal cytoplasm is thin, usually poorly delimited from the vacuole, and of low chromaticity. Although it has been shown to respond with plasmolysis to hypertonic solutions, the cell sap appears to pass readily from element to element through the sieve areas. The parenchyma cells show different degrees of ontogenetic and physiologic relation to the sieve elements. The companion cells of angiosperms are most closely associated with these elements; then follow the albuminous cells of gymnosperms and finally the phloem parenchyma and ray cells. The sieve elements are relatively short lived. The companion and albuminous cells die at the same time as the sieve elements.

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THE INTERNAL MECHANISM OF SPECIATION IN *GOSSYPIUM*

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INTRODUCTION

The last twenty years have seen great advances in the cytogenetics and taxonomy of the genus *Gossypium*. From the taxonomic point of view, controversial questions are now restricted to minor differences of opinion on relative taxonomic rank, while the major evolutionary pattern seems to be generally agreed upon. One outstanding major problem still remains, *viz.*, the origin of the amphidiploid species. Although there is general agreement on the parentage of the amphidiploids, there is a sharp cleavage of opinion as to the time when amphidiploidy occurred. Whether a satisfactory solution of this problem will be reached is to a large extent in the hands of the archaeologist and palaeontologist, though the cytologist, too, may be able to make pertinent contributions.

Cytogenetic research in cotton is now entering a new phase. In the absence of new and revolutionary techniques it seems likely that the immediate future will be concerned with the consolidation of knowledge already obtained and with attempts to apply that knowledge to practical ends—improvement of the cotton crop. One may expect a shift in the direction of research, particular emphasis being placed on the transference of potentially valuable economic characters from wild to cultivated species. It is therefore particularly important at this point to be sure that the theory of interspecific differentiation, which has been developed during the past twenty years, rests on a firm basis, since the success or failure of future cotton breeding programs may depend almost entirely on it.

The fact that a theory is generally accepted and has formed a keystone in the building of knowledge does not guarantee that the theory is correct. Nor, on the other hand, does it necessarily follow that all work based on the assumption of its being correct is invalidated if the theory eventually proves to be wrong. theory may be right for one set of data and wrong for another-the atomic theory is a striking example. It is important, however, to avoid converting a theory into a doctrine after it has ceased to offer a convincing explanation of more recent discoveries. If a theory is not elastic enough to embrace new data, it must be modified or discarded. It is the purpose of this paper to re-examine the commonly accepted theory of speciation in *Gossypium* and to point out certain genetic, cytological and breeding phenomena which it does not explain satisfactorily. In the writer's opinion these phenomena, while they do not necessarily invalidate the broader aspects of the theory, do show that it is incomplete and suggest a possible form of modification.

THE THEORY OF MULTIPLE GENE SUBSTITUTION AS A BASIS FOR SPECIATION

When the cultivated amphidiploid species, *G. barbadense* L. and *G. hirsutum* L., are crossed together, the hybrid exhibits heterosis to a marked degree and is apparently fully fertile. Meiosis in the F_1 pollen mother cell is normal insofar as regular bivalent pairing is acceptable as a criterion of normality. The F_2 generation, however, always shows a considerable depression in vigor; weak seedlings, sterile, and physiologically unbalanced types being common. The types which most nearly approach the F_1 in appearance again produce a medley of unthrifty types in the following (F_3) generation. Only those types which resemble most closely one or other of the parental species become stabilized under human or natural selection. A similar situation is found in hybrid progenies of the diploid cultivated species, *G. arboreum* L. and *G. herbaceum* L.

On the basis of his genetic studies of *barbadense* × *hirsutum* hybrids, Harland (15) first suggested an explanation of this "interspecific breakdown": "... The first step in the evolution of a species is the production of new multiple allelomorphs which differ from the old in their capability of being influenced by new modifier complexes, thus rendering possible the building up of new characters through natural selection of plus modifiers operating in the given direction". "... the modifiers really constitute the species and ... as a consequence of geographical isolation and natural selection evolution takes place by the evolution of new genes, by the production of new co-ordinated modifier systems and by the elaboration of new dominance mechanisms".

That each species has its characteristic "architecture" and behaves as a genetically co-ordinated system has been amply verified in *Gossypium* by Harland's own work and that of other investigators. His theory of speciation has proved to be a most valuable tool in the analysis of species relationships, without which a satisfactory classification would have been impossible. But in the writer's opinion it is questionable that the differentiation of species can be attributed primarily to the substitution of one "modifying complex" by another, unless the term "modifier" is used in a very specialized sense. Following the generally accepted usage of the term, a modifier is a gene which modifies the expression of another, the effect of the modifier being small, in comparison with the effect of the gene which it modifies, on the character under study. In that case the substitution of one modifier by another could involve only the substitution of each modifier by an allele at each corresponding locus, and if speciation depends primarily on this process, there is no valid reason why "good" daughter species should not remain cytologically homologous, locus by locus, throughout their respective chromosome sets. Any further differentiation would involve some degree of structural change. Harland apparently accepted this restriction, though, as will be shown later, evidence obtained in recent years is not compatible with it.

From his studies of interspecific hybrids involving the cultivated Asiatic species, *G. arboreum* L. and *G. herbaceum* L., and the wild African species, *G. anomalum* Wawra et Peyr., Silow (42, 43) considered that these species had evolved in a manner almost identical with that suggested by Harland to account for differences between *hirsutum* and *barbadense*. The F_1 hybrid, *arboreum* × *herbaceum*, shows normal bivalent pairing and is apparently fully fertile, but, as in the *hirstum* × *barbadense* cross, the F_2 consists of a range of types, most of which are inferior to each parent. The F_1 hybrids, *arboreum* × *anomalum* and *herbaceum* × *anomalum*, are only weakly fertile, and meiotic figures in their pollen mother cells show clear evidence of structural differentiation (5, 48). Nevertheless, Silow found that these hybrids were sufficiently fertile to enable him to undertake a very comprehensive study of the comparative genetics of the parent species. His conclusions agreed with Harland's: "... there is not any striking difference between the two cultivated Asiatic species as far as their main gene constitution is concerned. Practically all the difference lies in the modifier systems ...".

Anomalum was found to differ more widely from *arboreum* and *herbaceum* in respect to "main gene" constitution than these species differ from each other, and in modifier constitution it was "strikingly distinct from the two cultivated species, the hybrid progenies showing very much wider segregation than do crosses between the latter species". Silow's studies establish very definitely that the same basic mechanism is responsible for the differentiation of all species of *Gossypium* which are at least partially interfertile, and provide strong presumptive evidence that the same process has continued through the genus as a whole. It may be seriously questioned, however, that multiple gene substitution is as adequate a basic mechanism as he and many others, including the present writer, have supposed. In this paper an attempt will be made to show how the data accumulated in support of the hypothesis are capable of a different interpretation.

CRYPTIC STRUCTURAL DIFFERENTIATION AS A BASIS FOR SPECIATION

According to the theory of speciation by multiple gene substitution, the gross structural differentiation apparent in sterile or partially sterile hybrids must represent a secondary superimposed mechanism which may play a significant part in the isolation of species, but which cannot be directly concerned with the initiation of species differences. An exactly opposite point of view is held by Goldschmidt (10) who has suggested that species differences in *Gossypium*, among other genera, are attributable to different reaction systems determined by the structural patterns of whole chromosomes. *Is it necessary to accept either of these extreme hypotheses?* The writer first suggested (54) that genic and structural differentiation in *Gossypium* need not be considered as independent processes, and that a level of small scale structural differentiation could be envisaged which would be practically indistinguishable from purely genetic differentiation: "The frequency of *gross* structural changes (*i.e.*, inversions or changes in homology of sufficient magnitude to produce bridges) in interspecific hybrids... suggests the likelihood that *small* structural changes also occur. These cannot be detected cytologically though their presence may be inferred from the restricted chromosome pairing in interspecific hybrids. In crosses between interfertile species they will behave as Mendelian units and hence will be indistinguishable from true gene mutations. Small structural changes would account for the continuity of the process of differentiation which is seen at all levels of speciation".

That sterility in intraspecific hybrids, or in hybrids between closely related species, is probably attributable to small scale structural differentiation which would not necessarily be detectable cytologically was concluded earlier by Sax (40) and Muntzing (36) as a result of their studies in *Campsis* and *Galeopsis*, respectively. There is therefore a reasonable precedent for considering the possibility of a similar situation in *Gossypium* where, as in the above named genera, the chromosomes are small and few chiasmata per bivalent are produced at meiosis (46). Hutchinson and Stephens (27) recognized that there was no good reason to expect any sharp distinction between so-called "point mutation" and gross structural differentiation—the most likely situation being an intergrading series linking these two extreme cases. In the middle of the range it is likely that many small structural changes would be mistaken for simple allelic differences on the basis of breeding behavior. The difficulty of critical discrimination, even in the most favorable material (*Drosophila* salivary glands), has been emphasized by Raffel and Muller (38).

Stebbins (50, 51) has suggested the convenient term "cryptic structural differentiation" to cover the situation outlined above. From a general review of similar cases he has inferred that cryptic structural differentiation is commonly associated with speciation: "There is... a good deal of evidence now available that a large proportion, if not a majority of the structural differences between genomes, involve segments so small that they do not give the typical meiotic figurations such as multivalents and bridge fragments in structural hybrids.... Many of the numerous examples now known of diploid hybrids that are partly or wholly sterile in spite of nearly regular meiosis are best explained on the basis of the phenomenon of cryptic structural hybridity".

The present writer would go even further than this and suggest that in small chromosome types the production of markedly inferior F_2 progenies from fully fertile interspecific hybrids may also be attributable to cryptic structural hybridity, *i.e.*, to crossing-over and segregation of only partially homologous chromatids, leading to the production of deficient or otherwise unbalanced zygotes which would not necessarily be lethal nor completely sterile. In order for such an interpretation to hold it would of course be necessary to assume (*a*) that small deficiencies would not always behave as pollen lethals and (*b*) that crossing-over would occur fairly frequently between only partially homologous chromatids. With regard to (*a*), McClintock's evidence in corn (34) that a terminal deficiency so extensive as to include a knob, heterochromatic segment and half a chromomere in the ninth chromosome can be transmitted through the pollen removes any reasonable doubt that small deficiences can be carried in viable gametes. Furthermore, her work suggests that many so-called recessive mutants which are non-lethal may in fact be small deficiences which would be detectable only in very favorable material, *e.g.*, terminal deficiences in large chromosome types. With regard to (*b*) above, important information has been obtained at this station by Dr. M. S. Brown, which shows fairly conclusively that pairing between demonstrably non-homologous chromosomes can produce fertile gametes. Her evidence (8) in relation to the data of other investigators is summarized in Tables I and II. Referring to the tables, it can be seen that when the diploid *anomalum* (B genome) is crossed with the diploid *G. thurberi* Tod. (D_1 genome) the F_1 hybrid is completely sterile, and its meiotic figures show clear evidence of gross structural differentiation. The diploid *arboreum* (A_2 genome) gives a completely sterile hybrid on crossing with *thurberi*, and a partly sterile hybrid on crossing with *anomalum*. Colchicine synthesized amphidiploids, *arboreum* × *thurberi* ($2(A_2D_1)$) and *arboreum* × *anomalum* ($2(A_2B)$), are partly fertile and give preponderately bivalent pairing, indicating that pairing is chiefly between homologous chromosomes. But when the two amphidiploids are crossed together, their hybrid (A_2D_1/A_2B) is partly fertile, which is most readily explained as a result of regular bivalent pairing between the A_2 genomes and as a degree of regular pairing between the non-homologous B and D_1 genomes. An exactly similar situation was found in the cross (*arboreum* × *stocksii*) × (*arboreum* ×

anomalum), the multiple hybrid, A_2B/A_2E, being fertile, although the diploid hybrid, *anomalum* (B) × *stocksii* (E), was weak, unthrifty and sterile (47). The most likely explanation of the unexpected fertility obtained in these multiple hybrids is that the deficient or otherwise unbalanced chromatids resulting from crossing-over between B/D and B/E non-homologous "pairs", respectively, are covered by the presence of similar but not homologous chromatid segments in the normally pairing A_2 genomes. Deficiencies would therefore be masked in the resulting gametes by the normally balanced A_2 chromosome set. In the direct diploid × diploid crosses the deficiencies would be exposed and, presumably, since the resulting hybrids are sterile or partly sterile, would act as gametic lethals. Whether this is the correct interpretation or not, the foregoing data [illegible] extremely

TABLE I

MEIOTIC BEHAVIOR AND FERTILITY OF F_1 HYBRIDS BETWEEN CERTAIN DIPLOID SPECIES OF *Gossypium*. GENOME CONSTITUTION INDICATED BY APPROPRIATE LETTERS ACCORDING TO BEASLEY'S SYSTEM

F_1 hybrid	Genome constitution	Metaphase I	Fertility	Authority
1. *arboreum* × *thurberi*	A_2D_1	Half of total chromosomes associated in pairs. Obvious structural differences.	Sterile	Skovsted (48) Webber (64) Beasley (5)
2. *arboreum* × *anomalum*	A_2B	Over three quarters of total chromosomes associated in pairs. A few structural differences.	10% fertile	Skovsted (48) Silow (42) Beasley (5) Afzal *et al.* (2)
3. *anomalum* × *thurberi*	BD	Less than half of total chromosomes associated in pairs.	Sterile	Skovsted (48)
4. *arboreum* × *stocksii*	A_2E	Less than half of total chromosomes associated in pairs.	Sterile	Skovsted (48) Webber (64) Abraham (1) Beasley (5)
5. *anomalum* × *stocksii*	BE	1 nucleus analyzed by Beasley gave 5II 16I.	Sterile	Hutchinson cited Skovsted (47) Beasley (unpublished)

important bearing on the reliance one can place on "normal" bivalent pairing as a measure of cytological homology. Let us consider the situation which would exist if the amphidiploids cited had occurred in nature and if the original diploids had been lost. Since the amphidiploids give a partially fertile hybrid, we should almost certainly assume a reasonably close homology between their respective genomes—perhaps even suppose that their speciation was in the main associated with multiple gene substitution. In short we should apply the same reasoning as has already been applied to a similar situation, *viz.*, the differentiation of *hirsutum* and *barbadense*. But in the case of the synthetic amphidiploids we are fortunate in having the evidence of pairing behavior of the component subgenomes, *i.e.*, the original diploids, which indicates clearly that there is considerable structural differentiation. We can therefore no longer assume with any confidence that fertility of the *hirsutum* × *barbadense* hybrid is indicative of structural homology between the parent species.

TABLE II

MEIOTIC BEHAVIOR AND FERTILITY OF SYNTHETIC TETRAPLOIDS OBTAINED BY COLCHICINE TREATMENT OF CERTAIN OF THE DIPLOID HYBRIDS LISTED IN TABLE I

Synthetic tetraploid	Genome constitution	Metaphase I	Fertility	Authority
1. *arboreum* × *thurberi*	$2(A_2D_1)$	Approximates to "normal" bivalent pairing.	Female fertile, male sterile	Beasley (5) Harland (21)
2. *arboreum* × *anomalum*	$2(A_2B)$	Over three quarters of total chromosomes associated as bivalents.	Partly fertile male and female	Iyengar (28) Stephens (54) Brown (8)
3. *arboreum* × *stocksii*	$2(A_2E)$	Over three quarters of total chromosomes associated as bivalents. (Brown unpublished).	Partly fertile male and female	Stephens and Cassidy (60) Brown (8)
F_1 (1) × (2)	A_2D_1/A_2B	....	Partly fertile	Brown (8)
F_1 (2) × (3)	A_2B/A_2E	....	Partly fertile	Brown (8)

It might be argued that the above considerations are applicable only to hybrids between polyploid species, where the possibility of one sub-genome masking deficiencies in the other sub-genome would be at a maximum. In *Gossypium*, however, this cannot be considered a valid objection. There is considerable evidence, both cytological (5, 29, 46, 48, 52, 54) and genetic (42), that the "diploid" species, *arboreum* and *herbaceum*, either are themselves secondary polyploids or at least contain several segmental duplications.

EVIDENCE OF CRYPTIC STRUCTURAL DIFFERENTIATION IN *Gossypium*

The very term "cryptic structural differentiation" implies a difficulty in discriminating between this mechanism of speciation and a mechanism based on purely genetic differentiation. Furthermore, the general acceptance of the multiple gene substitution theory has not stimulated research in the development of critical tests of its validity. Evidence which has a bearing on the problem has to a large extent been collected for other purposes, and its significance has therefore escaped notice or else been underestimated. All that can be attempted here is to present rather scattered evidence obtained from cytological, genetic and breeding data, which in the writer's opinion is more consistent with structural differentiation than purely genetic differentiation, and to suggest methods of obtaining more satisfactory data.

Cytological Evidence

Bivalent Pairing in Fertile Interspecific Hybrids. Skovsted (46) noted that in pure species of Asiatic cottons, the maximum number of chiasmata formed from diplotene to first metaphase was three per bivalent, two being the most common number. In the interspecific hybrid, *arboreum* × *herbaceum*, the chiasmata frequency was less than in either parent species. Thus, with regard to the pachytene chromosomes, he states: "...it is the writer's impression that the doubleness of the pachytene strands was much more easily observed in the hybrid than in the pure species due to the fact that the threads were separated over larger distances".

Of the later stages (diakinesis and first metaphase) he says: "...all the partners must have formed at least two chiasmata which seems to be a common phenomenon in a normal diploid Asiatic cotton. In the interspecific hybrid the number of chiasmas is less, so that both rods and rings are common. A single pair is characterized by a conjugation so loose that most likely the chiasma is occasionally not formed which will result in univalent chromosomes as the second division indicates".

In F_1 hybrids from similar material, Webber (63) found occasional lagging bivalents and numerically unbalanced second metaphase plates. Beasley (5) frequently found a bridge and fragment at first and second anaphases in the interspecific hybrid, and also noted figures with four chromosomes associated.

In the interspecific hybrid, *hirsutum* × *barbadense*, both Webber and Beasley (*loc. cit.*) were of the opinion that meiotic chromosome irregularities occur more frequently than in the parent species, though evidence of structural differentiation was less than in the Asiatic diploids.

Silow (43), while noting the evidence of structural differentiation in the sources cited above, concluded that there was so little evidence of structural irregularity that the differences in the main must be referred to a genetic basis. While this may yet prove to be true, it now seems to the writer an unwarranted conclusion from the data available. Marked evidence of structural differentiation is not to be expected in species with small chromosomes and few chiasmata. The typical configurations observed at meiosis in pure species of *Gossypium* as a whole are ring bivalents resulting from the formation of only one chiasma per chromosome arm. The replacement of a ring by a rod bivalent in interspecific hybrids therefore means that in such cases one entire arm of chromosome has probably failed to pair, and this failure is best interpreted as evidence of reduced structural homology. Since so few chiasmata are produced in cotton chromosomes, however, the amount of reduction in number of chiasmata which is possible within the limits of regular bivalent formation is very restricted, and the degree of chiasma reduction in interspecific hybrids does not provide a good quantitative estimate of the amount of structural differentiation. Metaphase pairing, in fact, approaches an "all-or-none" process, so that reduced number of chiasmata could be the result either of a high or of a low degree of structural differentiation. It is theoretically possible, for instance, that some of the rod bivalents found in interspecific hybrids have only one pair of homologous arms, and, though this is not seriously considered as a likely situation, it is worth remembering that evidence from prophase studies or from genetic mapping of the chromosomes is at present quite inadequate to exclude it as a possibility.

Preferential Pairing in Allotetraploids Derived from Interfertile Species. It is clear that if two interfertile species have cryptic structural differences, the differentiation should be more readily observed in the allotetraploid obtained by colchicine treatment of their interspecific hybrid. Structural differentiation should here result in preferential pairing at meiosis, exhibited as a decrease in the number of polyvalent associations and an increase in the number of bivalent associations as compared with the corresponding associations in autotetraploids obtained from the parent species. Iyengar (29) presented the results of comparative meiotic studies in autotetraploid *arboreum*, autotetraploid *herbaceum* and the allotetraploid, *arboreum* × *herbaceum*, which prove conclusively that the parent species are structurally differentiated. Three strains of autotetraploid *arboreum* and two strains of autotetraploid *herbaceum* showed no significant differences in total number of metaphase associations per microsporocyte. Neither were significant differences found between three strains of allotetraploid *arboreum* × *herbaceum*. But the allotetraploids on the average produced 19.8 ± 0.13 metaphase associations as compared with an average of 17.9 ± 0.20 in the autotetraploids, which is a highly significant difference. Examination of Iyengar's tables shows that the difference is in the main attributable to the production of more bivalents and fewer polyvalents in the allotetraploids, as expected if the parent diploid species are structurally differentiated.

In the amphidiploid cottons, *hirsutum* and *barbadense*, it was found (5) that the F_1 hybrid after doubling with colchicine had an average of 15.2 quadrivalents at metaphase as compared with an average of 17.5 and 18.1 quadrivalents in *hirsutum* (doubled) and *barbadense* (doubled), respectively. The data as a whole are therefore consistent in showing that both diploid and amphidiploid species are structurally differentiated.

Now a little consideration leads to the conclusion that even the above comparative studies of auto- and allopolyploids yield only a minimal estimate of the actual amount of structural differentiation of the parent species. Owing to the small number of chiasmata produced in cotton chromosomes, a large proportion of the metaphase chromosomes are represented as bivalents in both auto- and allopolyploids. Since structural differences are present, it is extremely likely that in the allopolyploids the majority of these bivalent associations are paired homologues from the same parent species. If this should be the case, structural differentiation may be much greater than the increased number of metaphase associations in allopolyploids might lead one to suppose. It is impossible to test this situation by cytological methods, as individual chromosomes can not be distinguished from each other with existing techniques. It is possible, however, to test it genetically with appropriately marked stocks, as will be indicated in a later section of this paper.

Comparative studies of polyploids involving Asiatic cottons (*arboreum* and *herbaceum*) and *anomalum* also yield significant information. There has never been any doubt that these species are structurally differentiated, since the F_1 hybrids at first metaphase produce an average of 10 to 12 bivalents out of a possible 13 bivalents, and quadrivalents, trivalents and univalents are common (48). Beasley (5), in studying similar material, concluded that four or more structural differences exist. Afzal *et al.* (2) found about the same number of bivalents as had previously been reported by Skovsted and other workers, but no higher associations were observed. They mentioned that Iyengar had found trivalents and quadrivalents in material identical with that used in their own studies, and also that chromatin bridges and fragments were present at first anaphase, and chromatin bridges at second anaphase. Of particular interest was their finding that the amount of structural differentiation in the interspecific hybrids (*arboreum* × *anomalum*) varied according to the strain of *arboreum* parent used. It seems to the writer that this could be interpreted as indirect evidence for the existence of small-scale structural differentiation, even within the *arboreum* species. However, it is probable that part of the differences observed could be attributed to the effect of different environments.

The gross structural differentiation, exhibited as polyvalents, univalents and chromatin bridges, which is evident in the above studies is insufficient to explain the marked preferential pairing found in allopolyploids synthesized from hybrids of the parental species. It has been noted (52) that in allotriploids of the constitution A_2A_2D, the most common situation at first metaphase is that the *arboreum* chromosomes pair regularly as 13 bivalents, leaving the 13 chromosomes from the other parent unpaired. In the allotriploid obtained by crossing autotetraploid *arboreum* with *anomalum* it was expected, since the F_1 diploid *arboreum* × *anomalum* is partly fertile, that the situation in an autotriploid would be approached. (Skovsted (46) found six trivalents and additional higher polyvalents in an autotriploid Asiatic cotton). Actually an average of less than two trivalents per pollen mother cell was observed (54), which approaches the situation in the allotriploids examined earlier.

The allotetraploid *arboreum* × *anomalum* has been synthesized by several workers (3, 28, 54). There appears to be general agreement that it is considerably more fertile than the undoubled diploid hybrid, and that pairing is almost entirely between homologues from the same parent species, since it breeds true on selfing. The only published data on its meiotic pairing is given by Iyengar (28) who found 22.67 bivalents out of a possible 26. Preferential pairing to this extent is much greater than can be accounted for by the restricted amount of gross structural differentiation observed in the diploid hybrid, and the obvious conclusion is that the Asiatic (*arboreum* and *herbaceum*) and *anomalum* genomes are structurally differentiated throughout on a relatively minute scale, that meiotic pairing in the diploid hybrid therefore involves only partly homologous partners but does not entirely lead to the production of inviable gametes, and that pairing in the allotetraploid is almost entirely between homologous chromosomes, or at least that the gametes resulting from this type of pairing have a strong selective advantage.

Genetics and Breeding

Reduction of Crossing-over Between Linked Loci. Whatever the basis of interspecific differentiation in *Gossypium*, it is well established that interfertile species have many genetic loci with homologous series of alleles in common. This similarity in structure may be extended to the few linkage groups which are known.

The most striking example of the latter is the "Red-Cluster" linkage group (45) which is apparently common to the intersterile A and D genomes. In interfertile species the existence of common linkage groups offers a means of testing cryptic structural differentiation in the region between the linked loci. In interspecific backcrosses the linked genes should have a lower crossover value than expected on the basis of linkage tests conducted in intraspecific material, if structural differentiation reduces the chances of pairing within this region. In studying lint color inheritance in Asiatic cottons, Silow (44) found that lint color (Lc_1) was linked with leaf shape (L). Within *arboreum* the crossover value was 28.7%, but in *arboreum* × *herbaceum* backcrosses it was significantly lower (24.5%). When this reduction in crossover value is considered in relation to the cytological evidence of structural differentiation between these species, it would seem to have considerably more bearing on the speciation mechanism than Silow (43) attributed to it, though it is only fair to state that his opinion at the time was shared to some extent by the present writer.

Another linkage group which provides suggestive though not critical information is the "Green lint–Crinkle" linkage (17). Harland transferred the Crinkle gene from a White lint–Crinkle *barbadense* stock to a White lint–Normal *hirsutum* stock by successive backcrossing until all other visible *barbadense* characters had been eliminated and the Crinkle gene gave clear cut monofactorial segregation with its Normal allele in *hirsutum*. The transferred Crinkle was then tested against a *hirsutum* mutant, Green lint, and the two loci were found to be linked with 5% crossovers. One of the crossover types, Green lint–Crinkle, was maintained by selfing for several years and eventually used by Hutchinson (24) in further studies of the Crinkle allelomorph system. In an intra-*hirsutum* cross, in a backcross family of 305 plants, Hutchinson recorded 10% crossover values[1] between Green lint and Crinkle, *i.e.*, a looser linkage than determined by Harland in the same original material. A possible explanation of this discrepancy is that *barbadense* and *hirsutum* chromosomes

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[1] An F_2 family examined at the same time gave approximately 5% crossovers, but this value was obtained only after transferring more than half of one of the crossover classes to a non-crossover class on the grounds of misclassification, and must therefore be regarded as suspect. Before adjustment, crossovers in F_2 and backcross both approximated 10%.

are structurally differentiated between the Green lint and Crinkle loci. If this is so, then on transferring Crinkle to a White lint–Normal *hirsutum* type, Harland brought in a section of *barbadense* chromosome which lies between these two loci. On crossing and backcrossing the transferred White lint–Crinkle to Green lint–Normal, crossing-over would be partially suppressed between these loci, but what crossovers occurred might reduce the length of the interstitial non-homologous segment and replace it by a homologous *hirsutum* segment. Consequently the crossover type, Green lint–Crinkle, would show a looser linkage in this region in subsequent test crosses with *hirsutum* stocks.

Skewed Backcross Ratios. The most striking feature in the progenies of interspecific hybrids is the failure to obtain clear cut genetic ratios in the segregations of genes which are known to be allelomorphic on the basis of intraspecific tests. Instead, the normal range of each phenotypic class is greatly extended and there is often considerable overlapping, with the result that the expected monofactorial segregations become blurred and cannot be classified. According to the multiple gene substitution theory of interspecific differentiation, the blurred segregations are attributed to the segregation of modifiers — admittedly a satisfactory explanation. Another feature which has received less emphasis is the skewed ratios obtained in the interspecific backcrosses. Although a common feature, the skewed backcross ratio is not so universal as the blurred F_2 segregation, but tends to be associated with specific genes or the chromosomes which carry them. They are most frequently found in transferring genes from one rather distantly related species to another, *e.g.*, in the transference of Asiatic Red from *arboreum* to *barbadense* (18), of several independent genes from *anomalum* to *arboreum* (42), and of Normal allele of Crinkle from *thurberi*, and petal color (anthocyanin) from *aridum*, to *barbadense* (22). In all these cases, when the backcross families are sufficiently large in size, there is a marked deficiency of the class containing the gene from the donor parent. The deficiency cannot be due to the effect of the introduced gene itself because with successive backcrosses the normal 1 : 1 ratio tends to be more closely approached. Neither can it be attributed to modifier segregation because the distinction between genetic classes is relatively clear cut. It has usually been noted that the skewed ratios in the first and second backcrosses are accompanied by partial sterility and even aneuploidy, but these factors should have no differential effect on the ratios obtained unless the chromosome carrying the introduced gene is directly concerned. The writer prefers a more specific explanation along the following lines:

Partial sterility in early backcrosses implies that the chromosomes of the parental species are structurally differentiated, for which there is independent evidence in all the cases cited above. Deficiency of the introduced gene in backcrosses suggests that it is carried on an only partly homologous chromosome, or chromosome segment, the gametes containing which are at a selective disadvantage as compared with the gametes containing the corresponding chromosome from the recurrent parent. Further, the fact that in successive backcrosses the ratios tend to become "normal" suggests that crossing-over is occurring between the partly homologous chromosomes and hence, under the influence of selection, progressively removing "foreign" chromatin in the neighborhood of the introduced gene. The extent to which the gene becomes freed from its immediate neighbors should depend on the degree of structural differentiation between the chromosomes of donor and recurrent parent in this region.

If this explanation is acceptable when applied to species which are known to be structurally differentiated, then the occurrence of similar skewed ratios in crosses between species which are supposed to be structurally similar may be regarded as thought-provoking evidence. The best example is provided by the transference of the Crinkle gene from *barbadense* to *hirsutum* (13). The relevant data are analysed statistically below:

	Combined ratio		X^2 (1:1)	X^2 Heterogeneity
	Normal	Crinkle		
1st backcross	166	98	17 5, P very low	5.5, P (2) = 0.05–0.10
2nd backcross	27	24	0.2, P = 0.50–0.70	4.9, P (5) = 0.30–0.50
3rd backcross	186	172	0.6, P = 0.30–0.50	0.7, P (2) = 0.70–0.80
4th backcross	9	10	0.05, P = 0.80–0.90	....

It can be seen that there is a general tendency for the crinkle class to be deficient until the fourth backcross, but that the deficiency is statistically significant only in the first backcross where it amounts to as much as 25%. The writer has recently confirmed this marked selection against an introduced Crinkle locus in a first backcross of *hirsutum* to *barbadense, i.e.*, in a reverse direction to Harland's transference, and also found similar though less striking evidence of selection against introduced genes at the leaf-shape locus. The interspecific hybrid *hirsutum* "Narrow" leaf (L^O) × *barbadense* "Intermediate" leaf (L^E) was backcrossed both to *hirsutum* "Broad" leaf (l) and to *barbadense* "Intermediate" leaf (L^E). In the backcross to *hirsutum* the L^E/l class was deficient, while in the backcross to *barbadense* the L^O/L^E class was deficient, which suggests that it is the leaf-shape chromosome from the donor parent which is at a selective disadvantage in either case.

Selective elimination of this nature has not been found in several other monofactorial differences which have been studied, but this, of course, does not prove that selective elimination is only of rare occurrence. It is hardly to be expected that, if it occurs, it would be detected in studies of single gene segregations except when the marked chromosome segment from the donor parent is at considerable selective disadvantage, *i.e.*, probably considerably differentiated in structure from its partner in the recurrent parent. Probably in the majority of cases the selective disadvantage would be so slight as to be obscured by chance fluctuations in the expected 1 : 1 ratios. It is, however, entirely possible that the effects of many small structural differences would be cumulative and hence detectable as a more rapid elimination of the donor parent genotype as a whole than would be expected on the basis of random recombination and segregation in successive backcrosses. An experiment to test this possibility is in progress at this Station at the present time.

The examples of skewed ratios which have been cited give clear evidence of selection against a chromosome segment introduced from the donor parent. The evidence may be regarded as critical, since the skewness disappears with continued backcrossing as expected or the skewness is reversed when the direction of backcrossing is reversed. In addition to these cases there are other suggestive examples in the literature which should be re-investigated. Harland (12), in a series of F_2s and backcrosses from the cross yellow corolla × white corolla, found a consistent excess of white segregates in both intraspecific and interspecific crosses. The sole exception was in a selfed backcross (*hirsutum* white × *barbadense* yellow) × *barbadense* yellow where the skewness was reversed. The reason for the general excess of white segregates is unknown, but its reversal when the white gene is brought in from the donor parent is interesting and possibly significant. Hutchinson (23), in studies of corolla color in Asiatic cottons, found that a backcross of the type F_1 (*herbaceum* yellow × *arboreum* white) ♀ × *arboreum* white ♂ and its reciprocal gave an excess of white segregates, the deviation from the expected 1 : 1 ratio being greater when the F_1 was used as pollen parent. Intraspecific crosses involving the same genes gave good approximations to monofactorial ratios. This evidence cannot be considered critical because Hutchinson found that in one interspecific F_2 family the ratios obtained were apparently affected by the age of the seeds planted.

More general evidence consistent with cryptic structural differentiation is available in breeding data. In interspecific crosses between *hirsutum* and *barbadense* three or four backcrosses are sufficient to "complete" transference of a gene from donor to recurrent parent, *i.e.*, "complete" to the extent that the gene segregates normally, giving clear cut monofactorial ratios. At this stage of transference the backcross families are visibly so uniformly of the recurrent parent type, except for the introduced gene, that further selection is no longer effective (31):

"After the second or third backcross, the material is usually so near the backcross parent in appearance that large progenies possess little or no advantage. Small progenies become just as effective in eliminating any of the donor genotype which may remain . . .".

Now if the visible differences between *hirsutum* and *barbadense* are attributable to multiple gene substitution, each allele from the donor parent, other than the one being consciously transferred, should on the basis of random recombination and segregation appear in the diminishing order of 50%, 25%, 12.5% *etc.* of the plants in the successive backcross families. Therefore, if interspecific differentiation depends on large numbers of genic differences it would seem inconceivable that the number of donor genes retained by chance after only three or four backcrosses would be so low as to produce no detectable effect in the backcross family.

Clearly, as has been emphasized (31), much of the donor parent genotype still remains in the third backcross, but this does not alter the fact that the visible approach towards the recurrent parent is much more rapid than the visible differences between the parents would lead one to suppose. In short, it may be reasonably concluded that there is a rather stringent selection against genes from the donor parent, and this conclusion is supported by the rarity of gene exchange between mixed crops of interfertile species (27). Unfortunately no specific tests have been set up to test the rates at which genetic markers are eliminated in interspecific backcrosses, but in 1947 the writer had the opportunity to see some breeding material at the Pee Dee Station, Florence, S. C., in which the possibility of introducing *barbadense* genes into *hirsutum* by mass backcrossing was being tested by Mr. W. H. Jenkins. According to this system, individual selection is not practised in the early backcross generations, but pollen from as many plants as possible is mixed and used in crossing to the recurrent parent. The original parents differed in three known monogenic characters, *viz.*, petal color and pollen color, which segregate sharply even in interspecific backcrosses, and petal spot, which is rather variable in expression. In the third backcross with no selection, therefore, one would expect that 3 × 12.5%, *i.e.*, a little more than a third, of the plants should carry at least one of these introduced dominant genes. Actually these genes were found much more rarely than expected, and if the material is in any way representative of the usual situation in interspecific backcrossing, there can be no doubt that there is a strong selection against specific introduced genes or the chromosome segments which carry them. According to the multiple gene substitution theory, the only mechanism to account for differential elimination would be selection against gametes of zygotes which possess a "modifier complex" different from that of the recurrent parent, but it is difficult to see why any particular gene from the donor parent should have a markedly smaller chance of inclusion in the favored complex. Selection on this basis would be a long slow process and surely not detectable in monogenic class differences. A more logical explanation is that the chromosome carrying the introduced gene is at a selective disadvantage, or more precisely that the gene is carried on a differential chromosome segment. On this alternative basis it would be expected that in the interspecific hybrid both the non-crossover chromatids, carrying a differential segment from the donor parent, and crossover chromatids, carrying the consequent deficiencies and duplications, would be at a selective disadvantage as compared with non-crossover chromatids of the recurrent parent type.

Block Transference. As suggested in the previous section, structural differentiation would be expected to lead to a rapid elimination of donor parent genes in backcrosses, actually far more rapidly than a mechanism based on modifier differences. Conversely, conscious artificial selection of donor parent genes would be expected to maintain, against the forces of natural selection, whatever small blocks of non-homologous chromosome segments happened to be associated with the gene selected. In other words, backcrossing involving structurally differentiated species would imply introduction of blocks of linked characters rather than transference of individual genes, as expected by the multiple gene substitution hypothesis. These implications may have an important bearing on Harland's statement (19) that "in no case where a multiple allele series has been established is a dominant character common to both species", and further that "cases of identity (apparent or real) are practically confined to recessive genes only". There can be no doubt that when homologous series of alleles are found to be common to *hirsutum* and *barbadense*, the relative frequencies of the alleles are different in the two species (15, 19, 20). A similar situation is found in comparative studies (42) of the Asiatic cultivated species. But the evidence that Normal, or "wild type", dominants corresponding to certain mutants are different in different species is much less easily demonstrated. Hutchinson (24) did not find evidence for as many Normal alleles of Crinkle as Harland had formerly supposed, and with regard to the leaf-shape alleles, Stephens (55) pointed out that while tests of allelomorphism are relatively easy, tests of identity furnish a much more difficult proposition. In the case of the leafshape alleles a more critical analysis was possible. It was found (55) that three leaf-shape "alleles" occurring in *G. barbadense* var. *darwinii, G. hirsutum* var. *mariegalante* and *G. hirsutum* (Upland), respectively, were associated with phenotypically very similar leaf shapes. On transference to a common Upland background, however, their leaf indices were found to be significantly different statistically, and the difference was not reduced by further backcrossing. It might therefore have been concluded that different dominant alleles were characteristic of the three subspecies. This conclusion was not acceptable because it did not agree with the results of more critical developmental studies. It was found (53, 56) that the shape of the climax leaf in *Gossypium* is mainly dependent on three developmental processes: (*a*) the rate at which a juvenile undissected leaf is converted into a lobed adult leaf, which may be represented graphically as a "developmental track"; (*b*) the rate at which development proceeds along each track; and (*c*) the distance progressed along each track during the vegetative period of the plant's life history. Of these three processes only (*a*) is determined by the leaf-shape allele, while (*b*) and (*c*) depend predominantly on the flowering habit of the plant. It is therefore of considerable significance that process (*a*) was found to be almost identical for the three leaf-shape "alleles" under study, and the phenotypic difference between them could be wholly ascribed to processes (*b*) and (*c*). The most probable explanation is that the leaf shapes in the three subspecies are determined by identical leaf-shape genes, and that the differences between the transferred types were due to associated blocks of linked characters brought into the recurrent parent during backcrossing. Differences of this type can be analyzed only in certain favorable cases, and they show clearly that generalizations based on comparatively superficial phenotypic expressions may be rather misleading. It will be noted that the suggestion that the leaf-shape locus may be associated with a differential segment receives some support from the skewed ratios obtained in connection with segregation of this locus which have been cited earlier.

The idea that backcrossing one species to another, with selection of desirable characters from the donor parent, often results in the unintentional introduction of undesirable blocks of characters as well, finds some support in breeding experience. Unfortunately the data which could provide critical information on the nature of interspecific differentiation often go unrecorded and can be gleaned only in verbal discussions with plant breeders. Nevertheless, the considered opinion of people who have unrivalled opportunities for studying the segregation of characters in large populations must carry some weight. The synthetic amphidiploid, *arboreum* × *thurberi* (4), was successfully crossed and backcrossed to Upland (*hirsutum*) cotton. Analysis of fiber properties of the early backcross progenies showed that some of the segregates had greatly increased fiber strength (6) which could be attributed to certain fiber properties (low X-ray angle and narrow perimeter) inherited from the *thurberi* parent. Further work carried out independently in Texas and in North Carolina has shown that this property can be maintained through successive backcrosses, but there is an apparently strong negative correlation between strength and high yielding capacity (39) which has not yet been broken. In some lines plants with strong fibers tend to be less vigorous and to carry small abnormally shaped bolls; in others boll production is prolific, but the individual bolls are small and lint production low. In still other lines strength seems to be associated with partial sterility. Now physiological studies of the factors influencing fiber strength in *hirsutum* cottons (6) have shown that this character is affected in a highly complex manner by the general water relations of the plant, and that the factors which favor high strength are likely to be unfavorable to high yield. One might therefore suppose that the negative correlation between strength and productivity observed in the interspecific backcrosses is a necessary physiological consequence, and not attributable to genetic linkage. But in this case one would expect that the inheritance of high strength would be exceedingly complex from the genetic point of view, and that as a consequence it would be difficult to maintain strength through successive backcrosses. Actually a reverse situation is found, as strength is maintained and the proportion of strong fibered types suggests that rather few genes are involved. Physiological complexity and apparent genetic simplicity are not irreconcilable if the strength characters are carried on comparatively few differential chromosome segments which have been introduced by backcrossing into only partly homologous *hirsutum* chromosomes. In this case one would expect that the crossover chromatids, carrying differential segments, either might contain deleterious *thurberi* genes in blocks tightly linked with the strength genes, or might be deficient as a result of the initial crossovers. This interpretation finds some support in the fact that the chromosomes of *thurberi* and *hirsutum* are known to be structurally differentiated, and, further, in the observation (Dr. T. Kerr, personal communication) that when the selection pressure for strength is relaxed, strong fiber types are rapidly lost from the breeding material.

Block transference of this type is not limited to crosses between species, like the above, whose respective chromosome sets show gross structural differentiation. Parallel cases are found in crosses between *hirsutum* and *barbadense*. Knight (30) successfully transferred two dominant genes (B_2 and B_3) for Black-arm resistance from *G. hirsutum* var. *punctatum* Hutchinson to *G. barbadense*. The expected bifactorial segregation (3 resistant : 1 susceptible) in successive backcross generations was violently skewed (approximately 2 resistant : 1 susceptible), which was shown to be due to linkage between B_2 and B_3. Pooling the results of seven backcrosses, Knight arrived at a crossover value of 37.2%, but this value showed an overall trend, *i.e.*, it tended to increase with successive backcrossing. Inspection of Knight's data (Table 9) shows that the average crossover value calculated from the total of the first four backcrosses was 32.4%. In the fifth backcross there was an "apparent sudden loss of linkage", and the crossover value jumped to 48.5%. The average crossover value calculated from the total of fifth, sixth and seventh backcrosses was 41.3%. It seems likely that whatever causes were responsible for the increased chiasma frequency in the fifth backcross parents, they increased the chances of further crossing over in this region in subsequent generations. This is consistent with the hypothesis that the *punctatum* and *barbadense* parents had structurally differentiated chromosomes in the B_2 and B_3 region, and that the unexpectedly high crossover frequency in the fifth backcross parents resulted in the permanent replacement of a differential segment by one of the recurrent parent type.

In a more general way, evidence consistent with block transference is provided by the long continued attempts of cotton breeders to transfer desirable fiber characters from Sea Island (*barbadense*) to Upland (*hirsutum*) cottons. Of the extensive work carried on at Florence, S. C., Mr. W. H. Jenkins kindly informs me that it is "very difficult to carry over desirable characters of the Sea Island without carrying also undesirable characters such as low yield, low lint percent, small bolls, *etc.* Only by intensive selection at every stage has it been possible to carry desirable fiber characters, and it has taken long hard work to develop relatively few strains".

Blurred Monofactorial Segregations and Depressed Vigor in Selfed Hybrid Progenies. The multiple gene substitution hypothesis received its strongest support from the behavior of selfed progenies of interspecific hybrids. According to the hypothesis, multiple segregation of the genes comprising the "modifier complexes" of each parent species leads to a breakdown in their respective genetic architectures which have been built up by natural selection, with the result that most of the segregates are inferior to either parent. Similarly, effective genic action in each species is adjusted to the internal genetic environment built up by natural selection so that segregation in F_2 disrupts the appropriate *milieu,* and genic expression becomes highly variable.

There can be no doubt that this hypothesis furnished a satisfactory interpretation of these phenomena, but here it must be pointed out that it is not the only possible interpretation. Exactly the same behavior would be expected if the parent species were structurally differentiated in the cytological sense. On the latter alternative interpretation we should replace the rather vague concept of a genetic architecture built up of "constellations of modifiers", by a more precise cytological architecture depending on (*a*) the linear order and (*b*) the nature of the genetic loci throughout the chromosome set. In different species neither (*a*) nor (*b*) would be identical, though in interfertile species a rather close resemblance might be inferred. The interspecific hybrid would contain a complete genome from each parent, and the resemblance between corresponding but not strictly homologous chromosomes might be expected to lead to regular bivalent pairing. In *Gossypium* the low chiasma frequency results in a proportion of rod bivalents, *i.e.*, bivalents in which only two out of four chromatids are involved in a crossover. From such bivalents the chromatids delivered to the gametes would be of three types, (*a*) and (*b*), non-crossover types identical with the respective parental chromosomes, and (*c*), crossover types which, owing to structural differentiation, would carry deficiencies and their reciprocal duplications. Depending on the degree of structural differentiation between the parent species, gametes or zygotes carrying crossover chromatids would be at a selective disadvantage, and the most vigorous F_2 segregates would be those carrying the most non-crossover chromatids. That is, they would be phenotypically like either parent or like the F_1. Further crossing-over in later generations would tend to eliminate the F_1 type and to increase the number of parental type segregates. This is, of course, exactly what is found when interfertile species of cotton are grown together in mixed cultivation. The majority of the plants can be classified as belonging to one species or the other, the only exceptions being those which resemble the F_1. In experimental culture, segregates carrying crossover chromatids would be duplicated in respect to some segments and deficient in others, though in the zygote a considerable amount of mutual masking might be expected. It is possible that the "plus and minus modifiers" of the gene substitution hypothesis might be accounted for by duplications and deficiencies, respectively. In backcrosses all deficiencies would be masked by the complete chromosome set of the recurrent parent, and hence "modifier disturbances" should be greatly reduced. This is actually the case, and in fact many gene segregations which cannot be scored in F_2 give clear-cut segregations in the first backcross. Continued backcrossing should in the absence of conscious counter selection result in a rather rapid replacement of differential segments by those of the recurrent parent type.

Pseudo-alleles.; The evidence so far presented has shown that various cytological, genetic and breeding phenomena in interfertile species are consistent with the hypothesis that the species are structurally differentiated. The problem may also be approached in another way, *viz.*, in a search for evidence of the mechanism by which specific chromosome segments are becoming structurally differentiated. In order to consider this aspect it is necessary to review briefly some developments in general genetic theory.

The great majority of mutant alleles are at least partly recessive, *i.e.*, the heterozygote between wild type and mutant allele shows either complete dominance or intermediacy. Comparatively rarely, cases occur where the heterozygote shows a new character different from either parent—the "alleles" appear to have a complementary effect. This situation is found in certain long multiple allelic series, *e.g.*, in the *A* and *R* loci in maize (9, 49) and the elytra pattern series in *Harmonia* (61). Certain cases of complementary "allelism" have recently been shown not to be due to allelism at all, but to the interaction of non-homologous but closely adjacent loci, *e.g.*, the Yellow-green/Pale Yellow series in maize (34), the Scute series (41) and Star/Asteroid (32) in *Drosophila* and the anthocyanin series (65) in cotton. Since the pseudo-alleles, in spite of their complementary interaction, appear to retain a basic similarity in function, it appears likely that they have arisen as repeats of an originally single locus (32, 58) in a manner very similar to the origin of Bar and Double-bar (7) in *Drosophila.* Such an interpretation is not new, as both Haldane (11) and Muller (35) had suggested earlier that duplication offers a possibility for the development of new loci. The significant feature is that pseudo-alleles resemble independent genes and differ from true alleles in the fact that they produce a complementary effect in the heterozygous condition. In short, they behave as would be expected if they represented the first stage in the development of cytologically and biochemically new loci. If such should be the case, the production of pseudo-alleles would represent a highly important evolutionary step, since it would afford a means whereby structural differentiation and the development of new characters could be accomplished simultaneously. As such it might have a greater evolutionary significance than "point mutation", as the latter in itself provides no mechanism for the origin of new loci and in many cases (62) leads to the inactivation of loci already present.

Considering now the situation in cultivated cottons, it is to be noted that out of 17 independent gene-controlled characters in Asiatic cottons listed by Hutchinson and Silow (26), anthocyanin pigmentation and possibly also Crumpled are controlled by "alleles" which give complementary effects (59, 65). In the cultivated amphidiploid cottons, out of 13 characters studied (excluding duplicates), Crinkle and Corky are both controlled by complementary "alleles" (24, 57, 59). Of these four complementary "allelic" series, one (the anthocyanin series) is known definitely to be pseudo-allelic (65), and it is suspected that the other three may have a similar basis. It may well be, therefore, that about

10 per cent of the known loci in cotton are in the process of forming new daughter loci, *i.e.*, the first step in structural differentiation.

A somewhat similar line of evidence for small scale structural differentiation is provided by complementary genes. In cotton, as pointed out by Silow (45), it is not easy to distinguish between duplicates (giving 15 : 1 F_2 ratios) and complementaries (giving 9 : 7 ratios). The difficulty, however, goes deeper than the dominance modification with which he was concerned. It is possible to trace the successive steps by which true duplicate genes like the chlorophyll deficiency genes in amphidiploid cotton (14, 16) may become converted into complementary genes like the corolla color (42), pollen color (26), glabrous lintless and hairy lintless complementaries (25). The clue to the relationship was provided by Silow's discovery (45) of a duplicated linkage group in the amphidiploid cottons. Anthocyanin (R_1) and Cluster (Cl_1) form a linkage group in the D sub-genome, and Anthocyanin (R_2) and Short branch (Cl_2) form a parallel group in the A sub-genome of the amphidiploid (AD) cottons. Genes and their mutants at the R_1 locus are similar to, but not identical with, those at the R_2 locus; Cluster and Short branch would probably be indistinguishable in otherwise isogenic stocks. The facts that the linkage groups correspond, that they are carried in different sub-genomes, and that the R_1 and R_2 loci have been shown to be homologous with loci carried by the corresponding diploid relatives (18, 22), remove any doubt that these loci have evolved from common ancestral loci. With amphidiploidy, both loci were duplicated. But they are no longer duplicates. Genes at the R_1 and R_2 loci, though similar, give complementary effects in combination; for example, the interaction R_2 Ghost Spot with R_1 Spotless to give Red Spot (42). Cluster and Short branch give complementary Normal branch in combination (45). In short, these complementary "duplicates" bear exactly the same relationship to true duplicates as pseudo-alleles bear to true alleles, *i.e.*, they have diverged in function.

Further evidence of this relationship is provided by Silow's comparative studies (42) of anthocyanin pigmentation in *arboreum* and *anomalum*. In *arboreum* the production of a red spot in the petals depends on the interaction of two pseudo-alleles, Spotless and Ghost Spot (65). In *anomalum* a similar interaction is found, but the Spotless and Ghost Spot loci are carried in different chromosomes. Silow found that *arboreum* Ghost Spot and *anomalum* Ghost Spot were either identical or allelomorphic, while *anomalum* Spotless was a duplicate of *arboreum* Spotless. He assumed that a recessive allele was carried by *arboreum* at a locus homologous with *anomalum* Spotless, so that according to his interpretation the constitution of a red spot in *arboreum* and *anomalum* would be $(R^G R^S)$; r^O and $(R^G r^O)$; R^S, respectively. There is, however, no good reason to assume this structural homology. It is quite possible that the *anomalum* Spotless locus is carried in a translocated segment, which would be more in accordance with the fact that backcross ratios were skewed, and also with the independent evidence that occasional quadrivalent associations are found at meiosis in the interspecific hybrid. In this case the r^O symbols in the preceding formulae could be replaced by "blanks". Of particular interest was Silow's finding that the *anomalum* Spotless gene, though it resembled *arboreum* Spotless in giving a complementary interaction with Ghost Spot, was not identical with it. In *anomalum* Spotless a small amount of anthocyanin is present throughout the petal which interacts with the yellow pigment to give a "gold petal" or bronzing effect. In *arboreum* Spotless no anthocyanin is produced until the flower fades. Here again there is the suggestion that "duplicates" have undergone a slight divergence in function, and further that duplication and complementary interaction may be intimately related.

With regard to corolla color, Silow (42) found that a full yellow color depends on Y genes at three independent loci being present simultaneously. When any one of the Y genes is replaced by a recessive, Y^P, the corolla is pale yellow. Any two different pale yellow types are therefore complementary with each other giving a full yellow hybrid. On selfing, a ratio of 9 full yellow : 7 pale yellow is obtained in F_2, but since the different pale yellow types are usually distinguishable from each other, the ratio can be further broken down into 9 full yellow : 3 pale yellow (parent type A) : 3 pale yellow (parent type B) : 1 near-white (double recessive). The pale yellows can therefore be considered either as complementaries or as independent genes, according to the accuracy of scoring. However, as Silow has indicated, there is some reason to believe that the three *Y* loci were originally duplicates. This receives some support from the chemical evidence (37) that full yellow petals contain a mixture of three anthoxanthin pigments, gossypetin, quercetin and herbacetin. Quercetin and herbacetin are isomeric in chemical structure, and gossypetin may be regarded as an internally compensated "hybrid" of the other two. The writer's preliminary and unpublished studies of the three pale yellow complementaries suggest that they differ from full yellows and from each other only in the relative proportions of these three pigments. It may therefore be inferred that the nature of the gene-controlled processes associated with the three complementary loci are closely related, *i.e.*, probably had a common origin. Do these complementaries represent a transition between duplicates and independent genes, since they share some of the characteristics of both?

The glabrous lintless complementaries give a normal hairy linted phenotype in combination, and the F_2 segregates in the ratio 9 hairy linted: 7 glabrous lintless. The hairy lintless complementaries are similarly related. The glabrous lintless complementary pair is distinguishable from the hairy lintless pair by the fact that the former removes all hairs from the vegetative surface of the plant, while the latter removes only the seed hairs. Crosses between glabrous lintless and hairy lintless therefore give independent segregations in F_2 (9 hairy linted : 3 hairy lintless : 4 glabrous lintless). Were it not for their differential action on vegetative hairs, all four loci would be regarded as complementaries, any two of which on crossing together would give 9 linted : 7 lintless in F_2. Is it possible that the difference between the lintless complementary pairs represents the final stage in a transition from complementary to independent genes?

The data which have been considered may be summarized as follows: There is a certain amount of genetic and chemical evidence that in *Gossypium* a mechanism exists by which small scale structural differences (repeats) and the production of qualitatively new loci can occur simultaneously. Studies of complementary genes suggest that they may possibly be interpreted as transition stages between duplicates and independent genes. The importance of this mechanism in the speciation of the genus has yet to be assessed, but it must at least contribute to the structural differentiation which has been found by independent cytogenetical investigations.

METHODS OF TESTING FOR CRYPTIC STRUCTURAL DIFFERENTIATION

As pointed out earlier, the evidence reviewed in this paper is almost entirely incidental and not obtained as a result of experiments set up specifically to test the existence of structural differentiation. From the taxonomic point of view it is probably of little significance whether species distinctions are attributed to multiple gene substitution or to cryptic structural differences, but from the economic standpoint it is an issue of prime importance. Upon it depends the degree to which a plant breeder can hope to transfer a character of potential economic value from one species to another. If species differences are mainly attributable to multiple gene substitution, the problem is chiefly a matter of growing large enough progenies to secure the required combination. Alternatively, if species are structurally differentiated, there is no way available at present to open the "surprise parcels" of good and bad characters which may be transferred with every differential segment. It is therefore important to devise more precise methods of testing the nature and extent of interspecific differentiation.

The first method is available to all cotton breeders, since it consists merely of recording the rates at which various marker genes from the donor parent are lost in successive backcrosses. Mass backcrossing and reciprocal backcrossing afford particularly favorable material for this type of investigation.

The second method is based on the testing of linkages. Few linkage groups are known in cotton, but those which are known are common to two or more interfertile species. By comparative studies of crossover percentages between the same linked loci in intraspecific and interspecific backcrosses it would be possible to determine whether the apparent linkage is closer in the latter case, as expected if the chromosomes of the parent species are structurally differentiated within the limits of the tested region.

The third method depends on the preferential pairing expected in allopolyploids synthesized from fertile interspecific hybrids. As shown earlier, comparison of meiotic associations in allo- and autotetraploids provides a qualitative test of structural differentiation, but gives a very poor quantitative estimate. By using stocks from two interfertile species which carry the same genetic markers, allo- and autotetraploids can be synthesized which have the same genetic constitution in respect to the marker, *viz.*, *GG gg*. They will differ in the fact that in the allotetraploid the chromosomes carrying *G* will be from one species and those carrying *g* from the other, while in the autotetraploid all four chromosomes will be from one species. Preferential pairing will be indicated if the proportion of *gg* gametes produced by the allotetraploid is significantly less than the proportion produced by the autotetraploid, and the "preferential index" obtained should furnish a quantitative estimate of structural differentiation in the chromosome carrying the marker locus. It should be noted that the test is comparative and independent of agreement with the theoretical polyploid segregation. Of the various cytological factors (33) which influence the latter, *viz.*, mode of pairing, proportion of quadrivalents, chiasmata frequency, position of the locus relative to the centromere, the last named will be identical in both types of tetraploid, and the other three will be determined primarily by differential affinity which is the object of the test. The partial sterility of the tetraploids, and the consequent difficulty of obtaining progenies of adequate size, can be avoided to a great extent by using recessive (*gggg*) *hirsutum* as the tester stock in place of a multiple recessive autotetraploid. The sterile hybrid, *hirsutum* × tetraploid *arboreum*, can be obtained easily (52) and is very vigorous.

SUMMARY AND CONCLUSIONS

The evidence presented in this review shows clearly that multiple gene substitution, as suggested by Harland, is not sufficient to explain the cytological, genetic and breeding phenomena encountered in critical studies of fertile interspecific hybrids and their progenies in *Gossypium*. Studies of F_1 hybrids and of allotetraploids synthesized from them have provided evidence of cytologically detectable structural differentiation. Since few chiasmata are formed at meiosis in cotton species in general, it is clear that only relatively gross structural differences are likely to be observed. Such studies, therefore, do not provide a reliable quantitative estimate of the total amount of structural differences present. Recent evidence from studies of synthetic amphidiploids casts doubt on the validity of "normal" chromosome pairing and hybrid fertility as indices of structural homology. The following genetic and breeding phenomena in hybrid progenies are more consistent with cryptic structural differentiation than multiple gene substitution: (*a*) reduced crossing over; (*b*) skewed backcross ratios and rapid elimination of "foreign" genes; (*c*) block transference of characters resulting from conscious selection of only one gene. The postulated multiple gene substitution mechanism received chief support from the depressed vigor and blurred monogenic segregations found in selfed progenies; both these conditions, however, are equally well explained by crossing-over between similar but only partially homologous chromosomes. The relatively high proportion of "alleles" and "duplicates" which give unexpected complementary interactions suggests that a mechanism whereby biochemically new loci and small scale structural differences may be developed simultaneously is operating in cotton.

The general conclusion to be reached from these studies is that both multiple gene substitution and cryptic structural differentiation have determined the building of species from the earliest stages onward. The importance of the latter mechanism has yet to be assessed, but almost certainly has been greatly underestimated in the past. It is important now to devise critical tests, some of which have been suggested in this review, in order to obtain more quantitative evidence. The results are not likely to invalidate the inferences of taxonomic significance which have been drawn from the multiple gene substitution hypothesis, but they may have an important bearing on evolutionary mechanisms and breeding technique. The success which the breeder can expect in transferring potentially valuable characters from one species to another will be inversely proportional to the frequency of small scale structural differences in his breeding material.

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ISOTOPES AS TRACERS IN PLANTS

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INTRODUCTION

Isotopes afford an extremely useful means for following the absorption and transport of materials in plants and for elucidating the metabolic reactions of plants. The potentialities of the tracer method were early recognized by Hevesy who with Paneth in 1913 (72) used radiolead* as an indicator to determine the solubilities of lead chromate and lead sulfide, and in 1923 (63) followed the absorption and translocation of radiolead in plants. He applied the tracer technique in the same manner that it is used today (63), described its advantages and suggested how it could be employed.

The early limitation of the tracer method to naturally occurring radioisotopes was removed by the discovery of artificially induced radioactivity by Joliot and Curie (83), and the use of stable isotopes as tracers was made possible by Urey's methods (146, 150) of separating D, N^{15}, C^{13}, O^{18} and S^{34} in usable quantities. Supplementary developments that have been particularly helpful are the production of uranium piles (nuclear chain reactors), cyclotrons and other particle accelerators, simplified mass spectrometers for measurement of stable isotopes, and commercial Geiger-Müller counters and electrometers for measuring radioactivity. Since the release of radioisotopes by the Atomic Energy Commission (see Science **103**: 697-705. 1946; and the catalog and price list of radioisotopes distributed by the Isotopes Branch, U. S. Atomic Energy Commission, Oak Ridge, Tenn., for lists of radioisotopes and the form in which they are supplied), it has become unnecessary to maintain elaborate high voltage particle accelerators for most tracer work, as neutron bombardment in the uranium pile produces satisfactory radioisotopes of almost all elements of biological interest. Deuterium and O^{18} are also available through the facilities of the Atomic Energy Commission. N^{15} and C^{13} may be purchased from Eastman Kodak Company; individual institutions no longer need maintain thermal diffusion columns for the concentration of C^{13}.

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* The term radiolead, radiopotassium, *etc.*, will be employed to designate radioactive elements as suggested by Joliot and Curie (83).

Prior to the war the elaborate instrumentation necessary to produce and measure isotopes greatly limited their use. Now these obstacles have been removed, and with a modest investment a laboratory can be equipped for work with isotopic tracers. The use of isotopes is expanding enormously, and this trend will doubtless continue.

In explanation it should be stated that this review makes no claim to be an exhaustive survey of the literature. Some journals from the war years have not been available; certain review articles which describe original papers in no greater detail than the current review have been omitted; the literature on animals, yeasts, molds and bacteria has not been covered; and unquestionably some papers which should logically be included have been overlooked. This paper does not purport to describe methods in any detail. Kamen's (85) and Hevesy's (68*b*) recent books, the compilation of papers edited by Wilson, Nier and Reimann (157) and the University of Wisconsin Symposium on Isotopes (150) serve as excellent manuals for those wishing to employ isotopes as tracers. The following are useful references on the general application of isotopes: (28, 56, 59, 60, 65, 67, 68*a*, 73, 84, 87, 90, 92, 97, 102, 119, 120, 124, 127, 128, 138, 139, 140, 152, 160).

THE METHOD AND ITS MODE OF APPLICATION

The isotopic tracer method is basically very simple. A compound is labeled with an isotope, added to a chemical reaction mixture or to a biological system and allowed to react. After a suitable period the reaction is stopped, and reaction products are isolated and analyzed for their content of the isotope in question. The reaction mechanism is then interpreted on the basis of the distribution of the isotope in the products isolated. Thus the basic elements of the quantitative tracer method are synthesis, reaction, isolation, analysis and interpretation. As a variation of this scheme, the qualitative distribution of radioisotopes can often be followed in intact organisms without resorting to isolation procedures.

Synthesis usually involves no particular difficulty when one is interested in the uptake, transport or metabolism of inorganic ions, but is frequently a major problem when organic compounds are being traced. C^{14} was supplied initially as barium carbonate only, but it is now possible to obtain it commercially from Tracerlab, Boston, through the facilities of the Atomic Energy Commission, incorporated into barium carbide, acetylene, sodium cyanide, methanol, methyl iodide and carboxyl-labeled sodium acetate and ethyl acetate. The Atomic Energy Commission will also furnish O^{18} as H_2O^{18} and deuterium as either D_2 or D_2O; deuterium makes an excellent label for carbon chains when incorporated into stable linkages. Methyl iodide, NaCN and $BaCO_3$ enriched with C^{13} are now available from Eastman Kodak Company for those wishing to use the stable carbon isotope; this company also supplies $N^{15}H_4NO_3$ and N^{15}-enriched potassium phthalimide. Although starting materials for syntheses are limited, efficient methods for the production of many labeled compounds have been worked out. Usually the syntheses have involved well known methods adapted to small scale procedures and modified to improve yields relative to the isotopically enriched starting material. A number of these syntheses have been published (96, 102, 124 section V, 150 page 161).

Biosynthesis may often be useful for the production of complex labeled compounds, compounds such as starch (95), glucose and other sugars (122) which cannot be synthesized by chemical means, and the naturally occurring form of optically active compounds. In addition biosynthesis of certain simple compounds can compete directly with chemical syntheses on a basis of yield and convenience. A notable example is the production of certain organic acids by microorganisms (86); fermentation mechanisms for the production of these acids have been worked out in detail so that the location of a carbon label is well defined. Well-defined biosyntheses also may be accomplished with isolated enzyme preparations. The fermentation of labeled sugar and isolation of the products will also yield labeled compounds. Higher plants synthesize a tremendous variety of compounds, and isotopic CO_2 supplied to plants in closed containers is reduced by photosynthesis and eventually appears in these compounds to label their carbon atoms. The chief disadvantage of such syntheses is that the isotopic carbon may be distributed in the compounds in an unknown fashion. After biosynthesis the labeled material must be isolated, purified and its identity established before use.

Although our considerations have centered around the incorporation of isotopic carbon into compounds, other elements can also be added as labels by biosynthesis, *e.g.*, P^{32} in phosphorylated compounds and N^{15} in amino acids. The most convenient agents for biosynthesis of N^{15}-labeled amino acids are microorganisms, such as *Escherichia coli, Proteus vulgaris, Azotobacter vinelandii, Torula utilis* and *Saccharomyces cerevisiae,* which can utilize ammonia as their sole source of nitrogen. Their high protein content decreases the interference by extraneous material in isolation procedures, and by starting with small inocula very little dilution of the N^{15} supplied is experienced.

Following synthesis, the isotopically labeled compound is allowed to react in the system under investigation. The experimental conditions at this step will vary widely—the compound may be administered through the root system, through cut stems or petioles, infiltrated into intact or excised leaves, injected, given as a gas or vapor, applied in a paste or solution, or added to tissue slices, minces, homogenates or cell-free solutions. After a suitable time is allowed for reaction, the system is inactivated and products are isolated. Again the methods employed will be diverse and will be dictated by the nature of the experiment. Although it is impossible to generalize on the subject of isolation of products, it should be emphasized that the compounds isolated must be carefully purified. Constancy of the specific activity of a compound distributed between sets of solvents is useful in establishing its purity. Isotopes can be measured in such great dilution that a small trace of impurity is detected readily and may confuse the interpretation of results. The isolated products are analyzed for their isotopic label, and both their total isotope content and their specific activity (atom per cent excess for stable isotopes) are usually reported. Semi-quantitative results may be achieved by less laborious procedures, such as the radioautography (49) or counting of strips (88) from filter paper partition chromatograms. These data permit the investigator to interpret the metabolic reactions undergone by the labeled compound administered.

COMPARISON OF STABLE AND RADIOACTIVE ISOTOPES

Both stable isotopes, concentrated above their natural abundance, and radioactive isotopes may be employed as tracers. The choice between the two is often plain; for example, stable N^{15} and O^{18} are chosen because the radioisotopes of these elements have very short half-lives. On the other hand, the radioactive isotopes of phosphorus and sodium, P^{32} and Na^{24}, are indicated, as only a single stable isotope of each element exists. The choice between H^2 and H^3, C^{13} and C^{14}, S^{34} and S^{35} may not be so clear but will be governed by such considerations as the analytical equipment available to the investigator, the dilution to be experienced, whether there is a desire to stock a compound requiring a time-consuming synthesis (stable S^{34} *vs.* radioactive S^{35}), and the necessity for following the material in the intact plant.

The advantages of the stable tracers can be listed as their indefinite stability, their lack of radiation hazard and their adaptability to accurate quantitative work. The limited number of concentrated stable isotopes available is offset by the fact that the ones available are of great biological importance; if others were seriously needed they could unquestionably be concentrated.

Radioelements can be formed of almost any element, and although it does not necessarily follow that they will all be suitable as tracers, many of them serve as excellent labels. The radioelements have the advantage that they can be measured at tremendous dilutions; dilutions much greater than a million fold are often practical, whereas with the stable isotopes one is normally limited to a dilution of about a thousand fold. Radioisotopes are generally easier to measure than are the stable isotopes, and the equipment required is relatively simple and inexpensive. It is impossible to detect the stable isotopes except by destroying the test plant and analyzing with a mass spectrometer (there are alternative methods for measuring deuterium, such as the falling drop and refractive index methods), whereas radioactive isotopes which emit sufficiently energetic radiations can be measured by placing a Geiger-Müller counter tube or an ionization chamber near intact parts of the plant. Radioautographs (48) will also establish the distribution of radioisotopes in the plant.

It should be noted that quantitative methods are inherent in the technique of using stable isotopes. Similar rigorous criteria, if regularly applied to the use of the radioactive tracers, would greatly enhance the value of much of the tracer work conducted. Qualitative studies are of course extremely useful, but the magic of the word " isotope " and the ease and speed with which experiments can be conducted with radioisotopes have tempted us to accept observations without critical appraisal. Too often a labeled compound has been administered, and all radioactivity subsequently measured has been attributed to the intact molecule of the administered compound, whereas a radioisotope is actually detected whether it be in the original compound or any of a multitude of others formed from it as metabolic products.

Undesirable properties of radioisotopes include their radiation hazards to personnel and the ill-defined but sometimes deleterious effect of their radiations on the tissue being tested. Since it was possible safely to handle multicurie amounts of radioactive materials in the production of U^{235} and plutonium during the war, it is obvious that with suitable precautions tracer quantities of radioisotopes can be used without undue hazard. The dangers must not be minimized, however, and each laboratory should establish strict rules for handling and disposal of radioactive materials. (The Isotopes Branch, U. S. Atomic Energy Commission, P. O. Box E, Oak Ridge, Tennessee, will supply on request its circular B-1, "General Rules and Procedures Concerning Radioactive Hazards"). The worker's chief protections are shielding, distance, limited time of exposure, avoidance of direct contact with radioisotopes, scrupulous cleanliness and vigilant monitoring of work areas.

It has been argued (10) that radiotracers may alter the charge on the cell membrane, change the polar properties of the lipoids in the membrane, and denature cellular proteins, thus invalidating experiments conducted with them. There may be limited injury when high concentrations of radioelements are used (1, 54, 105, 107), but it seems to be the general experience and consensus of opinion (20, 41, 66, 85, 105, 106) that radioelements in the limited concentration normally employed as tracers have little harmful effect on living tissues.

It is a basic assumption in tracer work that all the isotopes of a given element will be used in an equivalent manner; since their outer electron configurations are the same they should have the same chemical properties. It is not strictly true that they are chemically equivalent, for their slight differences are utilized in the concentration of stable isotopes by chemical exchange reactions (146, 151). These differences are small enough, however, so that our analytical methods are usually not precise enough to establish any biological separation of the isotopes. Thus preferential utilization of one isotope of an element does not constitute a problem in tracer work. To this general statement an exception must be made for deuterium and tritium. Deuterium with twice and tritium with three times the mass of hydrogen differ substantially from hydrogen and have distinctly different effects on biological systems. In dilute solutions D_2O has shown no influence on growth of *Aspergillus niger,* germination of conidia of *Erysiphe graminis tritici,* root growth of wheat, and oxygen consumption by wheat seedlings (42). D_2O in high concentrations has proved inhibitory to the inversion of sucrose, catalase activity, the photosynthetic reaction (43, 121) and other biological reactions (125, 126). Tritium shows even greater differences from hydrogen. However, in the concentrations sufficient for most tracer work neither D nor T should prove toxic. The problem of isotope fractionation remains in using D_2, DT, D_2O, DTO, *etc.,* but does not apply to the use of D or T as labels for a carbon chain.

ION ABSORPTION AND EXCHANGE

Isotopes are nicely suited to investigation of ion absorption and exchange, and their extensive use in this field has been very informative. Hevesy (63, 64, 65) demonstrated that radiolead was rapidly taken up by the roots of *Vicia faba.* When the roots were then immersed in a solution containing a high concentration of normal lead, most of the radiolead went back into the external solution. Copper would displace lead from the roots very effectively; cadmium, zinc, chromium, barium and sodium were distinctly less effective. This ready displacement of radiolead suggested that it was predominantly present in the roots in an inorganic dissociable form rather than in an organic complex. The absorption of lead by the plant apparently had no direct connection with transpiration. When dilute solutions of radiolead were supplied, the bulk of it was retained in the roots, whereas from more concentrated solutions proportionately larger quantities were transferred to the tops of the plants.

There have been many demonstrations of the ability of single cells and intact roots to accumulate ions against a concentration gradient. In early experiments (91) it was demonstrated that the accumulation of radiolead by *Valonia macrophysa* occurred in the protoplasm and that very little of the ion entered the vacuole; when the cells were killed radiolead passed into the vacuole. The accumulation of uranium, radium and actinium in the roots of peas and other plants (7), copper in immersed leaves of *Elodea canadensis* (100, 101), rubidium and phosphorus by barley roots (117), and rubidium, bromine (24, 75), sodium and potassium in *Nitella* cells (22, 23, 24) has been demonstrated. In these cases, as with the accumulation of lead by *Valonia,* the concentration occurred in the protoplasm. Concentrations higher than those in the bathing fluid may be attained in the protoplasm in less than a minute (24, 25). Initially the labeled ion is more concentrated in the hyaline protoplasm, but later the granules of the protoplasm attain a higher concentration than the hyaline portion (108). The stimulation of organisms by extremely low concentrations of heavy metal ions (oligodynamic action) has been attributed to their ability to accumulate ions (101).

The initial rapid uptake of radioactive ions has been explained as an exchange of inorganic cations between the cell and the external medium (22, 24, 81). This initial exchange may be followed by the maintenance of a rather stable concentration or some loss of radioions, this phase in turn being followed by a slow but steady metabolic uptake of ions. A subsequent loss of ions may result from injury to the cells. Compared to accumulation in the protoplasm, movement of ions into the vacuole is relatively slow. After several hours exposure the concentrations in sap and protoplasm may not be greatly different (77). Tests with radiobromine (22) revealed no rapid uptake initially by *Nitella;* perhaps the cells had no appreciable concentration of proper ions for exchange.

The accumulation of ions is influenced by the energy metabolism of the plant (74). The respiration and fermentation of the plant generates H^+ and HCO_3^- which can be exchanged for ions from the nutrient medium (21). Under anaerobic conditions, or in the presence of respiratory poisons such as cyanide, accumulation of radioisotopes of rubidium or bromide is greatly inhibited (75). The initial non-metabolic exchange has a very low Q_{10} (about 1.10), whereas later accumulation has a Q_{10} of about 2.2, indicating its dependence upon respiration (109). Initially a good share of absorbed tracer ions may be held by the roots, but soon they move into the shoots as well (26).

The absorption rate for potassium ions by barley roots was found to be independent of concentration above 0.005 M, and calculations indicated that only about $1 \times 10^{-5}\%$ of the ions striking the root surface were absorbed (116). Absorption and translocation of ions by young barley was almost as active in the dark as in the light over short periods (76). Other observations with tomato have indicated a greater uptake of P^{32} as phosphate during the day than during the night and greater at 26.5° C. than at 18° C. (156).

When carrier-free Rb^{86} and P^{32} as phosphate were supplied in about 10^{-9} molar concentrations to actively metabolizing barley roots, the ions were accumulated rapidly (117). When the metabolism of the roots was virtually halted by keeping them at 0° C., the Rb^{86} absorbed non-metabolically was readily exchanged for normal rubidium added to the bathing solution, but the P^{32} taken up was not readily exchangeable. The non-metabolic uptake was largely confined to the first few millimeters of the root tip, and in this region the concentration within the tissue was greater than the external concentration of the ions; similar results were observed with carrier-free radiostrontium and radioiodine (79). In these experiments the root hairs did not appear particularly active in accumulating the radiostrontium and iodine. Roots of barley killed by treatment with ether are still able to take up strontium and iodine, and the dead roots may actually "accumulate" strontium ions. The killed tissues at 0° C. lose their isotopic tracers to a normal solution of ions much more readily than do live roots. It is suggested (79) that an ion-binding substance is elaborated in protoplasm during its aerobic metabolism.

After radioions have been taken up by plant cells they are not lost readily when the cells are immersed in distilled water (109, 110). However, they may be displaced by normal ions in the external medium (19, 110, 115, 117). It is evident that under these conditions ions pass into the cell, while others leave the cell; thus it is possible to build up or decrease the concentration of a radioion in a plant cell without the plant experiencing any shift in the total quantity of the ion present in its radioactive plus non-radioactive forms. The exchange movement of ions is much less dependent upon metabolic processes than is the accumulation of ions (27). An ion may be displaced from a cell by a non-homologous ion; thus lithium, potassium, rubidium and cesium will displace sodium; the lithium is more active in displacing sodium than is sodium itself (110).

The mechanism of absorption and loss of ions in soil is considered by some to involve an exchange between roots and soil colloids which are in contact (80, 81). After barley plants were exposed for two to five hours to radiopotassium in their nutrient solution, the roots were rinsed and placed in various nutrients without radioindicators. The radiopotassium was removed better by monovalent than by divalent cations, and better by cations adsorbed on clay particles than by cations in solution (82). This greater activity of contact exchange by adsorbed cations did not hold true for anions; radiobromine was removed from roots better by bromine in solution than by bromine adsorbed on clays. If roots and clay suspensions were separated by a semipermeable membrane, the solutions were more effective than the clay suspensions in removing radiopotassium and radiosodium.

When C^{11}-labeled $KHCO_3$ solution was supplied to barley seedlings (118), the bicarbonate ion was absorbed by the root system. However, the potassium was absorbed at a much more rapid rate. This differential absorption supports the ion exchange absorption theory (109) as opposed to the concept of absorption of intact molecules.

An influence of one ion upon another is shown by the influence of boron upon the concentration and solubility of strontium in tomato leaves (142). These experiments (142) were conducted with strontium on the assumption that it is metabolized in the same manner as is calcium; the use of strontium is justified as a qualitative but not as a quantitative tracer for calcium. The use of P^{32} has revealed that phosphate is more rapidly absorbed by phosphorus-deficient than by normal tomato plants, although it is distributed in the same compounds in each case (18). Tomato plants furnished urea absorbed phosphorus faster than plants grown on nitrate.

The problems of ion absorption and exchange are met constantly by those interested in soils. Isotopic tracers offer an elegant means for determining the origin of ions taken from the soil by plants. Fertilization is complicated by the fact that materials applied to soil may be bound by soil constituents, and as a result there is no assurance than an element appearing in a plant arises from the fertilizer element applied rather than from the same element already present in the soil. By the use of isotopes a fertilizer element may be labeled and traced unequivocally into the plant.

Tracer studies with soils were pioneered by Ballard and Dean (5) who described a great variation in sorption of P^{32} by different soils. A series of Florida soils ranked the same in phosphorus-fixing capacity, whether determined by chemical analysis, by measurement of P^{32} in equilibrium solution or by uptake of P^{32} by oat plants (40). The fixation of phosphorus was related to the clay content of the soils examined (111). Tests (5) on the application of P^{32} at the surface, deep in the soil at root level, and uniformly mixed throughout the soil indicated the greatest uptake of P^{32} from the deep application. Field experiments indicated that wheat plants grown about three and a half months recovered 23.6% of the P^{32} added to the soil (9, 143, 144). Plants vary greatly in their ability to utilize "fixed" phosphorus of the soil (6); Sudan grass, for example, uses it more readily than tomatoes.

P^{32} as phosphate and radiopotassium washed into the soil with the equivalent of two and a half inches of rainfall penetrated into a variety of soils to a depth of only an inch or two (62). As grape vines had not shown any measurable response to phosphorus fertilization, P^{32}-labeled phosphate was used to test their assimilation of the element. P^{32} plus normal carrier phosphate added at a 15-inch depth was taken up more quickly and in about twice as great an amount as when added to the surface; both treatments were followed by heavy irrigation. After 11 days, 99% of the P^{32} applied at the surface was in the top six inches of soil (149).

The fixation of cations by soil may be altered by grinding the soil. RbCl was added to soils, and the soils were air dried and then leached with ammonium acetate to remove replaceable Rb. By thorough regrinding in a ball mill Rb was rendered exchangeable to the extent of 27% for one and 50% for another soil, as measured by a repetition of the leaching process (99).

The availability of nitrogen from decaying organic matter has been tested by growing soybeans on a N^{15}-enriched nitrogen source, grinding the plants and incorporating them into soil-sand mixtures (113). Soybeans were grown on this soil for 11 weeks and tested for their N^{15} content; this was a measure of the proportion of their nitrogen derived from that originally present in the soil and that arising from decomposition of the added plant residues. Soybean plants which were inoculated (total nitrogen, 2,148 mg.) picked up 283 mg., and uninoculated plants (total nitrogen, 912 mg.) picked up 282 mg. of nitrogen from the plant residues in the soil.

TRANSPORT OF NUTRIENTS

Hevesy's early experiments (63) indicated that readily measurable quantities of radiolead were transported from the roots to the stems and leaves of *Vicia faba* plants, even when concentrations of lead nitrate as low as 1×10^{-6} N were supplied in the nutrient solution. At this concentration about 60% of the lead was taken up from the solution within 24 hours; at 0.1 N only 0.3% was taken up. At low concentrations a great percentage of the lead was bound in the root, whereas at higher concentration a proportionately greater amount was transported to the tops.

Following the earliest reports on the transport of P^{32} in plants (65, 70) there have been several studies of the transport of this excellent tracer. When part of a root system is immersed in P^{32}-labeled phosphate and part in normal phosphate, the transfer of P^{32} to the roots bathed in normal phosphate is rather slow (68). P^{32} was detected in the leaves of red Mexican bean seedlings two and a half hours after its addition as phosphate to the nutrient solution; the rate of transport exceeded ten centimeters an hour (13). The P^{32} accumulated in the roots to rather high concentrations before moving to the tops (14). The migration of P^{32} from the point at which it was injected into bean leaves seemed to follow a diurnal cycle with greatest downward movement near 10 A.M., the least at 10 P.M. and the maximum upward movement near noon (15). In a variety of plants both the xylem and phloem appeared to function in the upward transport of minerals (58). In the cotton plant radiophosphorus moved from the leaf via the phloem in both an upward and downward direction. The rate of downward movement was greater than 21 cm. per hour (16).

The presence of P^{32} in the tips of six-foot tomato plants was observed 40 minutes after the radioisotope was added to the nutrient solution (2). Radioautographs indicated that the P^{32} was absorbed in considerable quantity by the pulp and seeds of green tomatoes, a tendency that decreased with ageing of the fruit. On a limited supply of phosphorus the plant withdrew phosphorus from the leaves and transferred it to the developing fruit.

Complete sections of xylem were removed from geranium and *Bryophyllum calycinum* plants, and then the roots of the plants were immersed in a labeled phosphate solution. The concentration of P^{32} in the tops after 17 to 40 hours was about the same with intact as with treated plants, indicating that phosphate is transported in the pholem (57). These apparently clear-cut data have been challenged because of the relatively long time which elapsed before samples were taken.

When an attempt was made (39) to confine the transport of P^{32} to the phloem of Hubbard squash plants, the movement of P^{32} seemed to be correlated with the movement of food in the plants. In other experiments phosphate containing P^{32} was applied to the leaves after the petioles had been scalded to render the phloem non-conducting; no P^{32} was transported from the leaves, even though the xylem was functional. However, if the leaves were flooded with liquid, P^{32} and considerable liquid were transported through the scalded petioles. Three hours after vacuum infiltration of P^{32} into the leaves of intact plants the radioelement could be found 90 cm. from the point of treatment. Transport was through the xylem, as it was not prevented by scalding the petioles. Additional studies on the transport of radiophosphorus (19, 147) and radiosodium (19, 112, 147) have shown that both elements pass rapidly in a lateral direction from wood to bark (147); the indications are that the xylem is the path of rapid upward movement of salt.

Radiosulfur supplied in the nutrient solution to sulfur-deficient wheat and barley initially was accumulated avidly by the leaves; later this sulfur was more uniformly distributed throughout the plant (148). The bulk of the sulfur moved to the grain during ripening. Sulfur dioxide labeled with radiosulfur was used to fumigate plants. Again the radiosulfur accumulated in the leaves, and, as when sulfate was supplied in the nutrient solution, the inorganic forms were rapidly incorporated into organic compounds. Corn plants which had been given adequate amount of sulfur did not take up the labeled sulfate nearly as rapidly as the deficient wheat and barley. Radioautographs (61) indicated that radiosulfur was rather uniformly distributed in the wheat leaf, but in the kernel there was a marked concentration of sulfur in the embryo and also in the periphery of the endosperm. The distributions of protein and radiosulfur were very similar.

The stable isotope C^{13} has been employed to trace the transport of the products of photosynthesis (123). When single leaves were enclosed, furnished with $C^{13}O_2$ and illuminated, the photosynthate carrying the C^{13} label was transported both up and down the stem and tended to accumulate at the points of most active growth. After killing a section of stem with hot wax, P^{32} as phosphate passed up the stem, whereas C^{13}-labeled photosynthate did not pass up or down through such a block. This suggested the phloem as the exclusive path of transport for the photosynthate.

PHOTOSYNTHESIS

The importance and complexity of the photosynthetic reactions present a constant challenge. Isotopic tracers have been extremely useful in extending our knowledge of this process.

Results of a series of experiments with the short-lived radiocarbon, C^{11}, led Ruben and co-workers at Berkeley to conclude that the first measurable product of photosynthesis is a complex compound rather than any of the simple ones suggested earlier. Products of the dark fixation of $C^{11}O_2$ by *Chlorella pyrenoidosa* carried most of the C^{11} in material precipitable by barium in 80% ethyl alcohol (135); virtually all of this C^{11} was present in carboxyl groups. Dark fixation of CO_2 was also observed with barley leaves that had been illuminated shortly before exposure to $C^{11}O_2$ in the dark (130).

When the complete photosynthetic reaction occurred in the light, $C^{11}O_2$ was fixed in –CHOH– groups as well as in carboxyl groups (134). An examination by the carrier technique for products of photosynthesis after exposure of *Chlorella* to $C^{11}O_2$ for very short periods, yielded no evidence for the following substances as early products of photosynthesis: formaldehyde, acetaldehyde, propionaldehyde, glycolic aldehyde, glyceric aldehyde, methanol, ethanol, glycol, glycerol, erythritol, glucose, sucrose, starch, hexose monophosphate, glycine, alanine, arginine, histidine, albumin, acetone, and the acids formic, acetic, propionic, butyric, oxalic, succinic, malic, citric, maleic, fumaric, glycolic, pyruvic, glyceric, tartaric, lactic, ascorbic, glucuronic, glutamic, aspartic and glutaric.

The photosynthetic product from *Chlorella pyrenoidosa* carrying the bulk of the C^{11} had a molecular weight in the range of 1,000 (136), as established by ultracentrifugation. It was proposed (132) that photosynthesis occurs by an initial dark carboxylation of a high molecular weight compound to give RCOOH and that this is followed by a reduction of the carboxyl group in a light reaction to yield RCHO and then RCH_2OH with simultaneous liberation of O_2 from H_2O. Subsequent carboxylations and reductions could build up carbohydrate chains which could be split from the compound of high molecular weight.

When C^{14}-labeled acetate is supplied to tobacco leaves, a part of that which is not respired is soon converted to a wide variety of compounds (89). Tests with sunflower leaves have indicated that the photosynthetic products are rather rapidly used in respiration (141).

Experiments with Mg^{27} have yielded no support for the postulate that a reversible oxidation reduction occurs between chlorophylls a and b in photosynthesis (129). Tritium, H^3, was employed to test whether chlorophyll acts as a hydrogen donor in photosynthesis by illuminating *Chlorella pyrenoidosa* in HTO plus H_2O, isolating chlorophyll from the cells and testing it for radiohydrogen. Although the experiments are open to the possible objection that isotope fractionation does not favor T uptake, still they are highly suggestive that chlorophyll does not function as a hydrogen donor (114).

The origin of O_2 in the photosynthetic reaction was long a subject of speculation. Its origin remained undetermined until use of the stable isotope O^{18} revealed that O_2 arises exclusively from water (137). *Chlorella* cells photosynthesizing the O^{18}-enriched water evolved O_2 with the same O^{18} content as that of the water. The observation has been verified (44) and also shown to hold true for the Hill reaction (78).

Attempts to induce photosynthesis by ruptured *Nitella* cells were unsuccessful both with the chloroplastic and non-chloroplastic fractions (50). The products from photosynthesis by intact *Nitella* in the presence of $C^{11}O_2$ were water-soluble.

The availability of C^{14} recently has given new impetus to investigations of the early products of photosynthesis. Gaffron and co-workers (51) have substantiated the observation that a rather high molecular weight compound of unique properties is an early product of photosynthesis and that the commonly postulated intermediates do not appear to be involved. They have isolated a fraction, soluble in hot alcohol and insoluble in benzene, from *Scenedesmus* cells exposed to $C^{14}O_2$ for a minute or less; this fraction composing less than 1% of the dry weight of the algal cells carries the bulk of their radioactivity. Characterization of the compound is incomplete, but it has carboxyl and hydroxyl groups, may have lactone rings and is of rather high molecular weight.

In contrast, Calvin and co-workers (12, 37, 38) have studied the dark reaction in *Chlorella pyrenoidosa* and *Scenedesmus* with the assumption that the dark reaction is in all likelihood the initial step in photosynthesis and a logical point for isolating first products of the reaction. Isolations from *Chlorella* cells, previously treated to favor accumulation of reducing products and then exposed for short periods to $C^{14}O_2$, have shown high concentrations of the isotope in malic acid and in alanine. The high C^{14} specific activity of the phosphoglyceric acid and triosephosphate fractions suggests the condensation of triosephosphates as a likely pathway for sugar synthesis (38). It is tentatively proposed that carboxylations similar to those involved in the formation of pyruvic, oxalacetic and oxalosuccinic acids are concerned in photosynthesis. This viewpoint has been challenged and the data interpreted as indicative of "respiratory" CO_2 fixation quite apart from any function in photosynthesis. The two groups of investigators are operating under distinctly different experimental conditions, and it is too early to judge the validity of their respective conclusions regarding the early products of photosynthesis. Certainly the current efforts should greatly expand our information on the photosynthetic reaction.

The distribution of C^{14} in photosynthesizing barley seedlings has been determined (4). Glucose isolated from barley plants supplied with $C^{14}O_2$ showed the highest C^{14} concentration in the 3 and 4 positions and the least in the 1 and 6 positions. The distribution of C^{14} in glucose from intact plants was distinctly different from that in plants without roots. The data are compatible with the idea that hexoses are formed from triose phosphates and that the terminal carbons of the trioses, which become carbons 3 and 4 of the hexose, have particularly high C^{14} activity (3).

HETEROTROPHIC FIXATION OF CARBON DIOXIDE

Heterotrophic fixation of carbon dioxide has been shown to occur in plant and animal tissues and bacteria (133, 160). Barley roots assimilated $C^{11}O_2$ (133). A specific system involved in such CO_2 assimilation has been obtained in a cell-free state from parsley roots (153). The formation of oxalosuccinic acid from alpha-ketoglutaric acid had been demonstrated earlier with animal tissue as a reversible step in the tricarboxylic acid cycle. It is not unlikely, in fact evidence suggests, that a cycle such as that found in animal tissue also occurs in plants. However, except for the experimental work cited (153), the data presented in support of a tricarboxylic acid cycle in plants are non-specific and scarcely convincing. A thorough investigation of possible intermediates, specific steps and the overall reaction with the aid of isotopically labeled compounds is needed for our acceptance or rejection of the cycle in plants.

BIOLOGICAL NITROGEN FIXATION

The radioactive forms of nitrogen have very short half-lives; the half-life of N^{13} is 9.9 minutes. This limits their usefulness, and the stable isotope, N^{15}, is much more satisfactory as a tracer.

It was observed (131) that the tops of barley plants exposed for 20 minutes to N_2 containing N^{13} picked up radioactivity with the N^{13} half-life. This was surprising, since non-leguminous plants are not generally credited with the ability to fix molecular nitrogen. Experiments with N^{15} indicated that no exchange reaction of appreciable magnitude occurred in the biological fixation of nitrogen (33). The N^{13} experiment was repeated (29), using N^{15} as the tracer and growing intact barley plants aseptically for periods of 42 and 56 days; inoculated and uninoculated clover plants were subjected to the same treatment. No fixation of nitrogen greater than the experimental error of the method was observed for barley or for uninoculated clover, whereas the inoculated clover plants fixed nitrogen actively. Calculations indicated that the discrepancy in results could not be attributed to the greater sensitivity of the radioactive tracer.

Numerous agents have been alleged to fix molecular nitrogen, and many of these claims have now been checked with N^{15}; N^{15} offers a much more sensitive and specific means of testing such claims than does the Kjeldahl nitrogen determination. The well established nitrogen-fixing agents, leguminous plants plus root nodule bacteria (29), the aerobic *Azotobacter vinelandii,* the anaerobic *Clostridium pasteurianum* and the blue-green alga *Nostoc muscorum,* all fixed large quantities of N^{15} (32). The questionable agents, aseptic germinating peas, free-living root nodule bacteria, cell-free preparations of *Azotobacter* (32) and ground pea plants (31), gave uniformly negative results. Excised nodulated roots from leguminous plants fixed nitrogen rather consistently, but excised nodules only occasionally (32, 98). In contradistinction to carbon dioxide fixation, which isotopic tracers have revealed to be widespread in nature, biological nitrogen fixation is apparently limited to relatively few living forms.

The root nodules have been accepted as the actual locus of nitrogen fixation in leguminous plants. Experimental proof of this point has been rather difficult to obtain, but the accepted idea has been verified by the use of N^{15} (31). Although much larger amounts of N^{15} were fixed when it was supplied to the roots, it is interesting that when N^{15}-enriched N_2 was supplied to the tops of the plants it could be detected in small amounts in the nodules. This indicates that in all probability N_2 in solution passed through an exposed section of stem through the root and into the nodule where it was fixed.

N^{15} has been useful in establishing certain of the physical constants characteristic of the nitrogen-fixing enzymes of the blue-green alga, *Nostoc muscorum* (36).

Experimental data accumulated on biological nitrogen fixation with N^{15} as a tracer have been summarized and evaluated recently (158). It has been possible to study the relative use of fixed nitrogen compounds and molecular nitrogen by supplying *A. vinelandii* with fixed nitrogen compounds containing normal nitrogen in the nutrient medium and N^{15}-enriched N_2 in the atmosphere above the medium. The organism can obtain its nitrogen exclusively from the fixed nitrogen or from the N_2 or can obtain part from each—final analysis of the cells indicates how much of their nitrogen came from each source. Such experiments have indicated that only ammonia and urea (urea is practically equivalent to ammonia because of the strong urease in the cells) completely suppress nitrogen fixation in cultures which have not previously been grown on fixed nitrogen. Nitrate and nitrite suppress fixation considerably; amino acids alone or in mixture only slightly (159).

If $(N^{15}H_4)_2SO_4$ is added to an aerated culture of *A. vinelandii* which has been fixing N_2 as its sole source of nitrogen, the fixation of N_2 is immediately (within one minute) and completely suppressed (35). When $N^{15}O_3^-$ is added there is no detectable uptake of N^{15} for 30 minutes, after which nitrate is assimilated at an accelerating rate. When $N^{15}H_4NO_3$ is added to cultures of *A. vinelandii* which have been grown previously on N_2, the ammonium ion is used up before the nitrate is assimilated. Even cultures which have been grown on KNO_3 up to the time of the addition of $N^{15}H_4NO_3$ use the ammonium before the nitrate ion (35). Apparently *A. vinelandii* cells which are dependent upon N_2 (or NO_3^-) as their sole source of nitrogen have all the enzymes necessary for the utilization of the ammonium ion to the exclusion of other nitrogenous compounds, whereas the cells must adapt themselves to utilize nitrate.

A. vinelandii cells were grown in aerated culture for 18 hours and then aerated for 90 minutes with O_2 and N^{15}-enriched N_2. Certain amino acids were isolated from the cells, and analysis revealed that the highest N^{15} concentration was in glutamic acid, the next highest in the aspartic acid fraction (30). Likewise when N^{15}-labeled ammonium ion was added to an aerated culture the glutamic and aspartic acids carried the highest concentration of N^{15}, and the distribution of N^{15} in the other compounds was very similar to that found when N^{15}-enriched N_2 was supplied (34).

All the data from N^{15} experiments with leguminous plants and the free-living nitrogen-fixing bacteria appear compatible with the ammonia hypothesis of biological nitrogen fixation. Further application of this tracer should give us greater insight into the intermediates in the fixation reaction and additional suggestions on the mechanism of the reaction.

METABOLISM OF NITROGEN IN PLANTS

Other than in studies of biological nitrogen fixation, N^{15} has received rather limited use in investigations of the nitrogen metabolism of plants. The administration of N^{15} as ammonia to buckwheat (154), to tobacco plants (155) and to sunflower (69) indicated that there was a rapid replacement of leaf protein nitrogen by simple nitrogenous compounds of the cell sap and that a continuous exchange occurred between free amino acids and those combined in proteins. This established in plants, as had been demonstrated earlier with animals, that a dynamic equilibrium exists among nitrogenous compounds. The demonstration with plants is necessarily less elegant than with animals because plants cannot be maintained in a condition of nitrogen balance as can animals.

METABOLISM OF PHOSPHORUS IN PLANTS

In addition to studies of ion absorption and transport in plants, P^{32} has been used to establish the mobility of the element during growth of the plant. After ten days in a culture solution, corn plants were transferred to a solution containing P^{32} as phosphate. The plants grew an additional 21 days, were harvested, ashed and analyzed for P^{32}. The lower leaves, which were almost entirely formed before P^{32} was added, had virtually the same concentration of P^{32} as had the new leaves formed entirely after the addition of P^{32} (70). Very similar results were observed with sunflower (71). It is evident that phosphorus is a highly mobile element in the plant, and the newly acquired phosphorus readily exchanges with that laid down earlier in the plant.

PHYTOPATHOLOGY

By growing tobacco in a nutrient solution containing P^{32} it has been possible to isolate tobacco mosaic virus with good recovery of the P^{32} (17, 94, 145). When this virus labeled with P^{32} was inoculated into leaves and allowed to remain for 12 days, the bulk of the P^{32} was recovered in non-virus components. The difficulties in using labeled virus were enumerated (145).

There was little difference in the total uptake of P^{32} by healthy and heavily rusted wheat leaves, but in the rusted leaves the P^{32} accumulated in the infected areas. This concentration of P^{32} likely occurred in the host cells, since the uredospores carried only low levels of P^{32} (55).

PLANT-GROWTH REGULATORS

The transport of herbicides has been followed very nicely by the use of I^{131} as a label. This isotope is an excellent tracer, for it can be obtained in high specific activity, has a reasonable half life, 8.0

days, and emits energetic radiations. I^{131} was incorporated into 2-iodo-3-nitrobenzoic acid and followed through bean and barley plants. The compound applied to the leaves of bean seedlings was translocated to the stems and accumulated mainly in the terminal buds and in the hypocotyls. When absorbed by barley leaves it accumulated mainly in the second leaf (161); the growth regulator was translocated somewhat less readily than in bean. Further studies (104) again indicated that oat and corn plants absorbed less of the growth regulator than did bean plants. However, this observation could not account for the differential effects of the compound on mono- and dicotyledonous plants, for at equal concentrations of 2-iodo-3-nitrobenzoic acid in the plant tissues the bean plants showed marked responses, whereas the corn and oat plants were unaffected.

In experiments such as those just described it is dangerous to assume that when 2-iodo^{131}-3-nitrobenzoic acid is applied to a leaf and I^{131} is found in another part of the plant, 2-iodo^{131}-3-nitrobenzoic acid is present in this other part of the plant; it is always possible that the compound may be broken down and the I^{131} transported in another form. Fortunately this possibility has not been overlooked, and data have been presented (103) to establish that in these tests the intact herbicide molecule migrated.

STIMULATION OF THE GROWTH OF PLANTS BY RADIOACTIVE ELEMENTS

Hevesy and Paneth (73) refer to early work which indicated that, although large doses of radium are harmful, small amounts distinctly improve the growth of plants. Gager (52, 53) observed stimulation of plant growth under certain conditions by Ra but was not convinced of its practical importance. Over 100% increase in the dry weight of pea plants has been reported when 1×10^{-11} g. of radium was added to six liters of nutrient solution; even 1×10^{-8} g. per six liters was not lethal (45). The conclusion is drawn that uranium, radium, actinium and thorium are necessary nutrients for plants and that potassium cannot replace other radioactive elements; the interchangeability of radium, thorium and uranium was not tested. Although some other stimulations (11) have been reported, the lack of control and replication in the experiments suggests need for their verification. Extensive tests of the claims are currently planned in this country; some of the preliminary tests on vegetable crops under field conditions here have shown distinct stimulation by radioisotopes. Additional reports have been published concerning the stimulation of plant growth by radioactivity (46), the influence of radioactivity on nodulation of leguminous plants (47), the assimilation of naturally occurring radioelements (7), the amount of naturally occurring radioelements in soils (8) and the influence of immersion of plants in radioactive spring water upon their subsequent growth and development (93).

POTENTIAL USES OF ISOTOPES AS TRACERS

The foregoing treatment necessarily has been sketchy with regard to any particular application of isotopic tracers, but the variety of problems in which they have yielded valuable information is suggestive of their potential future usefulness. Many problems not amenable to the classical chemical or physiological approach may be attacked successfully with the aid of isotopes.

Isotopes are elegantly suited for investigations of ion absorption, exchange and transport in the plant, and unquestionably they will be used extensively in such work. It is hoped that accompanying such studies there will be more emphasis on the elucidation of the specific function of the various mineral elements in plant metabolism.

Many problems in soil chemistry are ideally suited for the use of isotopes. At last a means is available to distinguish fertilizer elements added to the soil from those already present and to determine their respective rôles in the nutrition of the plant. Studies on the influence of various treatments upon the mobilization of bound elements in the soil and upon the function of microorganisms in this regard could be conducted readily with isotopic tracers.

There is no question but that isotopes will be of immense aid in untangling some of our most complex plant processes, such as photosynthesis and biological nitrogen fixation. Already encouraging progress has been made in determining the early products of the photosynthetic reaction, and information obtained with radiocarbon has forced us to discard postulated intermediates which have beclouded the photosynthetic picture for three quarters of a century.

With a disappearance of the early tendency to use isotopes for the sake of employing a fashionable technique rather than for the inherent value they might have for the problem at hand, it is anticipated that more solidly conceived and carefully executed work will be forthcoming. Studies involving the isolation and characterization of intermediates will be helpful in establishing the mechanism of respiration in plants, the occurrence or non-occurrence of an organic acid cycle in plants, and the mechanism of synthesis of carbohydrates, proteins and lipids.

SUMMARY

The recent release of radioisotopes as produced in the uranium pile, the availability of reliable equipment for measuring radioactivity, and the commercial concentration of stable isotopes have given great impetus to the use of isotopes as tracers. The radioactive isotopes excell the stable isotopes in ease of measurement, variety available, dilution in which they can be detected, and for detection in intact organisms, whereas the stable isotopes are favored for their stability, applicability for tracing H, N and O, and their lack of radiation hazard. However, the stable and radioactive isotopes should be considered as complementary tools.

Radioisotopes have revealed the great speed of ion penetration into cells, the ready exchangeability of ions inside and outside the cell, and the dependence upon respiration for ion accumulation subsequent to the initial ion exchange.

Isotopes offer a means for differentiating added fertilizer elements from those already present in soils in the investigation of the nutrition of plants. In addition they offer an excellent means for following the transport of elements and compounds in plants.

Application of C^{11} and C^{14} has forced the discarding of old hypotheses concerning the early products of photosynthesis. Two distinct postulates have replaced the earlier ideas, one favoring the early formation of a hydroxylated, carboxyl compound of high molecular weight, and the other implicating the organic acids, particularly malic, as early products of photosynthesis. With the aid of O^{18} it has been shown that H_2O is the exclusive source of O_2 in the photosynthetic reaction.

Whereas the use of isotopes of carbon has revealed the widespread occurrence of heterotrophic fixation of carbon dioxide, the use of N^{15} has confirmed the limited distribution among organisms of the ability to fix N_2. Data obtained with N^{15} have all been compatible with the ammonia hypothesis of biological nitrogen fixation. As in animals, the nitrogenous constituents of plants are not inert but are in dynamic equilibrium.

Phosphorus is a highly mobile element in the plant, as evidenced by the uniform distribution of P^{32} in old and young leaves of plants supplied P^{32} when nearing maturity. P^{32} has been used in investigating the changes which occur in tobacco mosaic virus injected into leaves.

The movement of herbicides in plants has been traced with I^{131}, and it has been demonstrated that the differential response of mono- and dicotyledonous plants cannot be explained merely on the basis that the monocotyledonous plants absorb more of the herbicide.

Literature has been cited advancing the claim that radioactivity stimulates the growth of plants, but the data are in need of extension and verification.

NOTE ADDED IN PROOF

Many notable publications describing applications of isotopes to studies of reactions in plants have appeared since this review was transmitted July 27, 1948. Particular progress has been made in the investigation of photosynthesis with C^{14} as a tracer. Extensive collaborative experiments with P^{32} have revealed numerous new facts on the binding and recovery of this element from soils. Whereas early tests suggested that addition of radioisotopes enhances the yield of certain truck crops, subsequent trials have been predominantly negative.

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TECHNIQUES FOR THE ISOLATION OF SINGLE MICROORGANISMS. II[1].

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INTRODUCTION

When the first review (Hildebrand, 1938) was prepared, emphasis was placed on isolation techniques, with particular reference to single microorganisms. Since then this phase of micrurgical science has been broadened, and, while emphasis in this article is still on the techniques of isolation, some attention will be paid to its logical sequents—inoculation and germ-free culture of microorganisms and of macroscopic organisms.

It is precisely true that when studying the mechanism of infection, inoculation is the counterpart of isolation. The inoculum must be controlled and be definite in quality and quantity, and it is conditioned by isolation technique. In this related field of interest it was demonstrated for the first time (Hildebrand, 1937) that a single isolated cell of the fire blight organism, *Erwinia amylovora*, is adequate and capable of inducing infection of apple and pear blossoms when introduced by means of a micropipette. By a modification of the same precision technique (Hildebrand, 1941, 1942), crown gall infections, caused by *Agrobacterium tumefaciens*, were readily induced by inserting or planting a single bacterial cell into small wounds on tomato. By still another technique of microinjection into living tomato plant cells it was demonstrated, on the one hand (Hildebrand, 1943), that crown-gall bacteria are incapable of inducing infection when inside the cells, whereas when aucuba mosaic virus extract was injected into single living cells of tomato plants infections resulted in as many as 50 percent of the trials. Thus inoculations in the cases cited were the sequents of isolation and in the science of cytopathology are dependent on the perfection of isolation techniques.

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[1] Supplement to article in The Botanical Review 4: 627–664. 1938.

In the science of micrurgy the germ-free culture of multicellular, macroscopic organisms is a recent outstanding development that has been initiated and largely developed at Lobund,[2] the Laboratories of Bacteriology at the University of Notre Dame. The papers presented at the first symposium on Micrurgical and Germ-free Methods held there in 1939 were published subsequently (Reyniers, 1943). Just prior to the publication of this report, Baker and Ferguson (1942) announced the success of an operative procedure on pregnant platyfish (*Platypoecilus maculatus*) that regularly yielded young free from contamination.

Reyniers and associates (Reyniers, 1943; Reyniers and Trexler, 1942, 1943) have employed the term "germ-free" for the growth of vertebrate animals free from all forms of microscopic and macroscopic contaminants, whether internal or external. Subsequently, Baker and Ferguson (1942, 1944) chose the term "axenic" from the Greek, which means "free from strangers", for their cultivation of platyfish for four months free from contamination. It is up to the reader to choose which term—"germ-free" or "axenic"—best describes "life without germs" for microscopic or macroscopic plants or animals. The writer prefers "germ-free" because of its prior use and simplicity. Germ-free animals provide the best means for developing an understanding of disease resistance and immunity in animals.

MICRURGY—A NEW SCIENCE

"Micrurgy" is the term for work on a microscopic scale (Hildebrand, 1943). Thus single-cell isolation technique is a phase of micrurgical science involving micromanipulative technique under a microscope. Two popular accounts have been published about this new science (Chambers, 1935; Battista, 1947). The former article stresses the fact that there is a practicable way of studying what goes on in living cells by microvivisection, and that this is accomplished with the aid of minute needles and pipettes of glass controlled by a micromanipulator. These fine microinstruments are so tiny they are invisible except under the microscope. In most arrangements the microinstruments work against the underside of the microscope slide which forms the top part of the moist chamber.

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[2] The first of the Lobund Reports (No. 1: 115 pp.) was published in 1946; its title is "Germ-free life studies". The second, entitled "The need for a nomenclature describing germ-free life and the techniques for maintaining it", was published in 1949.

Battista (1947) characterizes micrurgy as a comparatively new science involving the use of midget tools under the microscope for delving into the fascinating worlds of the infinitely small. He predicts on the basis of present progress that one day in this atomic age it may become a major science comparable to astronomy.

The Symposium on Micrurgical and Germ-free Methods (Reyniers, 1943) has charted some of the earlier developments in micrurgy in application to experimental biology and medicine. Much of the work delineated by Battista (1947) is in application to the physical sciences—a field of interest that has not been reviewed in any literature that has come to my attention.

Reyniers (1942) and Reyniers and Trexler (1942) have further enlarged upon another phase, that is, the control of cross contamination by mechanical barriers which revamp our basic concepts on the application of microbiology to medicine.

PURE CULTURE BY DILUTION METHODS

The pure-culture concept that a culture to be pure must consist "of individuals of which we know with certainty that all are descended from one single cell, and from one only" has been occasionally challenged.

The inadequacy of the poured plate technique advocated by McNew (1938*a*, 1938*b*) for procuring pure single-cell cultures was discussed by Riker and Baldwin (1939). In brief, it was pointed out that the ordinary poured plate technique, while generally useful, appears inadequate for critical bacteriological work, particularly where strain variations are involved. The common poured-plate technique is considered insufficient because: (*a*) unless special precautions are taken, many of the colonies which develop come from bacterial clumps; (*b*) a large percentage of the colonies may run together in commonly employed dilution; while many such mixtures are readily seen, doubtless a number of others are not visible; (*c*) practical experience has shown that important plant bacteria are not always separated by dilution plates, even with elaborate precautions.

Chapman (1945) gives his concept of a "strain" in bacteriology with the suggestion that strains "should only be considered as such when it is known that they are genetically unrelated". As was pointed out by Hildebrand (1945), our present knowledge and methods are inadequate for achieving such precision. The word "strain" usually connotes a physiological difference which is possibly genetic in origin but not feasible of ready demonstration as such by present methods.

PURE CULTURE BY SEMI-MECHANICAL METHODS

Pure culture by semi-mechanical methods places emphasis on the isolation instruments or isolators employed, as was detailed in the previous report (Hildebrand, 1938). Most if not all of the reports published during the past decade are modifications of the older published methods, ordinarily involving the spreading of spore suspensions on an agar surface and the use of a tool fastened somehow to the microscope to separate an isolated cell for transfer to a sterile medium. Several of the more important reports will now be briefly discussed.

DRY NEEDLE METHOD. Chilton and Wernham (1940) mounted a simple single-spore isolator needle on the condenser holder of a microscope for manipulating in an isolation chamber consisting of a Van Tieghem cell, glued to the center of a piece of wood the size of an ordinary microscope slide with a hole, of the same diameter as the inside of the cell, in the bottom. The point of the needle was centered in the microscope field by manipulating the arm holding it.

Martin (1943) employed a simple technique for isolating spores of various fungi from exposed slides in aerobiological work. First, under the low power of the microscope the location of a spore or group of spores was marked with a crow-quill pen and India ink. Second, the slide was then inverted over a Van Tieghem cell, and single spores were isolated by means of a micromanipulator, according to Hanna's Method (1928). Third, if spores germinated and produced sporulating cultures, they could be identified by their manner and kind of spore formation.

Tapke (1946) devised a rapid method for isolating single ascospores from apothecia. Application of alcohol to the outer and lower surfaces of apothecia caused instantaneous ejection of ascospores of various species of *Sclerotinia,* a method slightly modified from that taught him by the late H. H. Whetzel in 1917. Three Petri dishes containing nutrient agar were placed near the vial containing the apothecia. These dishes should preferably exceed the fruiting body only slightly in height and diameter and be opened in the spore cloud in quick succession. The last plate usually catches only a few spores in perfect position for isolation and quite free from contaminants. Very small apothecia were held face downward over the nutrient agar and rubbed with a needle moistened with alcohol to cause ascospore ejection.

CYLINDER LOOP NEEDLE METHOD. Lambert (1939) announced a spore isolator combining some of the advantages of the Keitt (1915) and La Rue (1920) methods. The instrument is essentially a "biscuit cutter" mounted vertically in a threaded brass plug, screwed on the microscope in place of the oil-immersion objective. In practice it was found advisable to make the "cutter" about ¾ mm. in diameter or slightly less than the microscope field under the low power. The cutter can be centered by bending the wire so that it will mark off a circle concentric with the low-power field of the microscope. While possessing the mechanical precision inherent in the La Rue (1920) method, it also is adapted for lifting out the agar disc bearing the spore so that transfer can be made with no danger of the "spatula" touching the surrounding agar.

McLean (1939) described two improved types of cutter plunger, attachable to the nose-piece of a microscope, for cutting out portions of agar bearing single spores. One is fitted with a safety spring, and both are supplied with a closely fitting ramrod for pushing out the agar by air compression.

Georg (1947) reported a simple and rapid method for obtaining monospore cultures of fungi. For isolating spores 3 μ or larger, a small conical glass tip was made from a length of glass tubing with the larger end fitting round the lens of the low power objective (6 to 7 mm.) and the smaller end approximating the microscopic field as seen through the lens (1.5 to 1.8 mm.). The glass tube was heated to redness and drawn out to the required shape. A thin ring of plasticine was fitted round the lens of the low power objective, and to this the sterile conical glass tip was affixed. When the spore is centered in the microscopic field, the objective can be lowered so that the small end of the tip cuts a disk of agar containing the spore. The disk is then removed and transferred by a sterilized needle.

Northcraft (1948) described and illustrated a rapid method of single cell isolation involving an isolator fastened to the microscope condenser. A piece of sterile 4.0 percent agar, about 10 mm. square and 0.5 mm. thick, was placed in the center of a 22 × 40 mm. coverslip. The cells to be isolated were gently streaked with an inoculation needle onto the surface of the agar square. The coverslip with the inoculated agar square was then inverted over and centered in the window of the Lucite slide, a drop of glycerine at each end of the coverslip anchoring it to the slide. The mount is placed on the microscope stage and examined under the low (about 100 ×) power. Each cell can be studied under high power. When a cell is selected, the condenser is raised until the glass tube shadow shows. After centering, the cells are ringed by raising and lowering the condenser. The slide is then placed agar side up under a binocular dissecting microscope where cell and agar plugs are removed by a microscalpel for transfer to a test tube culture medium.

INDIA INK DROPLET METHOD. The India ink method, discovered by Ehrenberg (1838), is fully reviewed by Niklitschek (1939). The ink may be rubbed up with distilled water in a depression of a white porcelain plate, using a clear glass rod. Addition of ink should stop when the color of the ink creeping up the margin changes from brown to jet black. Trituration, however, should continue for at least 15 minutes longer. A "dry method" is employed for isolating blood parasites with a drop of blood mixed with a drop of India ink. The so-called "wet method" was used for isolating protozoa by employing equal amounts of culture fluid and India ink in mixture.

MICROCOLONY METHOD. De Zeeuw (1943) produced a special nutrient medium to obtain single-spore cultures of *Agaricus campestris*. A large number of uncontaminated spores were smeared on an agar slant with a sterile brush and allowed to germinate and grow for one to two weeks. After a solid mycelial mat had formed, the culture was killed by immersing the tube in boiling water until the medium was completely liquid. The dead mycelial mat was then removed aseptically and the sterile medium used for hanging drops on cover slips over Van Tieghem cells. Instead of only five percent germination by the usual methods, from 30 to 50 percent of the single spores of two commercial varieties of mushrooms isolated and placed on this medium germinated in replicate tests.

MISCELLANEOUS METHODS. Harding (1939) developed a simple instrument for dissecting minute animal organisms. It is based on the principle of the pantograph and is attached to the microscope for focusing without using the hands. With it one can make a permanent mount under one coverslip of all the appendages dissected from one small crustacean.

Lloyd (1940) designed an object-marker for the microscope which occupies one of the apertures in the triple nosepiece. When centered, the marker is brought down by the focusing adjustment into contact with the coverglass. Then the marker is given one complete rotation, causing the point to describe a circle whose diameter depends upon the adjustment previously made. A selected splinter of carborundum makes a neat circle which lasts for a considerable time.

Olson (1942) describes a device for marking fields on microscope slides. It consists of removable tips fitted into the nosepiece of the microscope which when centered to the area and inked can be lowered until the end of the tube touches, leaving a ring of ink around the field to be marked.

Hegre and Blount (1945) have perfected a chin-operated focus adjustment to free the hands when both are required for the manipulation of the object under the dissecting microscope.

MICRONEEDLE AND MICROPIPETTE INOCULATORS. Storey's (1933, 1938, 1939) series of micrurgical investigations is the most outstanding that has been conducted on insect transmission of plant viruses. In 1933 he illustrated his methods of inoculation by means of a special microneedle and micropipette, using semimicrurgical methods—performing the operations under the dissecting microscope. The reader is referred to these papers for further details.

Black (1941) transmitted the aster yellows virus mechanically by micropipette from insect to insect, that is, the diluted juice extract from the bodies of viruliferous leafhoppers held at 0° C., when injected into the insect abdomens at dilutions as high as 1–100 in 0.85 percent NaCl solution, was effective in transmitting the virus to healthy leafhoppers. This feat has thus far not been accomplished from diseased to healthy plants by mechanical means.

White and Braun (1941) demonstrated crown gall (plant cancer) perpetuation by bacteria-free tumor tissues. For initiation of galls in the first place the host cells must be under the influence of the crown gall bacteria (*Agrobacterium tumefaciens*) until they acquire the capacity for autonomous growth. Tissue fragments from the occasional crown gall tumors on sunflower stems were successfully cultured *in vitro* on special nutrient media and shown to be bacteria-free. When implanted by micrografting into healthy plant tissue, some typical crown gall tumors developed.

Blakeslee (1944) described a method whereby small embryos from artificial cultures or from mature small seeds may be grafted onto normal stock of *Datura stramonium* with protection against evaporation by means of gelatin capsules. By this method of micrografting, the excised embryos, which fail to develop roots, developed into useful seedlings.

PURE CULTURE BY MECHANICAL METHODS

Several new instruments have been added to further extend the extreme diversity in micromanipulator design. Chambers (1940) reviewed recent developments of the micromanipulative technique and its applications. His article is a shortened and somewhat modified account of a chapter prepared by him and M. J. Kopac for McClung's (1937) "Handbook of Microscopic Technique".

Micromanipulators

PNEUMATIC PRINCIPLE. At the time the first report was prepared (Hildebrand, 1938) the writer had no definite citations on the new micromanipulator which was being developed by De Fonbrune in France. First note to attract attention was made at the University of Montreal by Brunel (1938) who mentioned the De Fonbrune pneumatic micromanipulator in reviewing recent progress in the science of micrurgy. Several important articles have been published by De Fonbrune (1932, 1935, 1937). In the latest article (De Fonbrune, 1937) the new pneumatic micromanipulator, the receptor (isolator holder) and the microforge are described along with the use of the latter for the preparation of microinstruments. In Part 1 a detailed description is given of the pneumatic micromanipulator and how it functions for the operation of the receptor which holds the micro-instruments. Part 2 deals with microforge and its makeup. Part 3 gives the techniques in the use of the microforge for preparing a wide variety of microinstruments. The price quoted then for one micromanipulator, one receptor and one microforge was between 10,000 and 15,000 francs.

SIMPLE LEVER PRINCIPLE. Buchthal and Persson (1936) devised a micromanipulative apparatus which permits accurate work at magnifications up to 500 diameters. This micromanipulator is similar to the Emerson (1932) apparatus but operates with several concentric ball-and-socket joints with one lever only. It uses the screw-and-slide principle for gross movements. In operation a lever turns a ball. The length of the lever can be varied with the length of the. lever ratio to the offset from the ball, determining the reduction in the movements performed.

MISCELLANEOUS DESIGN. In 1938 Hanson (1938) announced a new design of micromanipulator which was capable of allowing motion of the isolator (quartz needle or other fine tool) for small distances and in any direction. This instrument is of multiple design. According to the report the conditions attained are: a considerable ratio between the motion of the operator's hand and the needle, good rigidity, freedom from backlash, freedom from wear (a defect of the Barber (1904, 1914) instrument) and ease of construction. In a test for lost motion it was found that the average difference in micrometer settings amounts to about one wave-length of light.

SCREW-AGAINST-SPRING PRINCIPLE. Pyke (1939) devised a simple micromanipulator mounted on a rigid base plate. The base plate consists of angle steel measuring three inches by three inches by ¾ inch and seven inches long. The movement of the needle or pipette is controlled by two rigid T-shaped levers, A and B, the needle being attached to lever B. The fulcrums on which these levers rock are formed by steel balls ⅛ inch in diameter which engage in grooves filed on the vertical members.

HYDRAULIC PRINCIPLE. Cailloux (1943) described a new hydraulic micromanipulator composed of two units, micromanipulator and receptor, which holds the microtools. The manipulator is essentially composed of a lever mounted on a universal joint to which are attached three pistons of small diameter. When the lever is moved by the operator, the liquid is displaced by the pistons in each of the three tubes connected to the receptor. The liquid finally acts upon each of the three flexible diaphragms on the receptor connected to the main shaft bearing the microtool. The pistons are not coupled in rectangular coordinates. Instead, the connecting rods are all attached to the same point on the main lever and, with the pistons, seem to form the edges of a pyramid. Thus in a straight horizontal movement, the three pistons displace equal amounts of liquid in the tubes. The membranes of the receptor are connected similarly. This method of grouping the pistons and membranes simplifies the mechanism of the instrument and leaves a minimum of articulations, thus avoiding loss of movement through play between parts.

The Cailloux hydraulic micromanipulator offers several advantages: (*a*) all movements of the microtools are controlled by a single knob; (*b*) positive response without lag of the microtools; (*c*) parasite vibrations of the manipulator are not transmittted to the microtools; (*d*) to obviate inversion of the image in a microscope, the receptor can easily be placed in such a way that the microtool seems to be the continuation of the lever on the manipulator; (*e*) the ratio of demultiplication can be varied at will; (*f*) it is suggested that the instrument user does not need previous training to use it expertly; (*g*) in its manufacture very few parts need to be machined with great precision.

Of the new instruments described, the writer has tested and operated only the Cailloux instrument located at the University of Montreal.

Isolators

Several designs of isolators, or instruments used in micromanipulative technique, have been used in the past. Some special machines have been invented for fabricating microinstruments, of which the L. G. Livingston "pipette puller" is an outstanding example. Undoubtedly more progress has been made in the past decade in the invention of instruments for the fabrication of microtools than in the microtools themselves.

MACHINES FOR FABRICATING MICROINSTRUMENTS. De Fonbrune (1937) describes in detail his new instrument, the microforge. After giving a detailed description of the pneumatic micromanipulator in Part 1, Part 2 deals with the microforge and its makeup, and Part 3 gives the techniques in the use of the microforge for preparing a wide variety of microinstruments, including microrods, microelectrodes, microloops, microscalpels and micropipettes.

Comandon and De Fonbrune (1938) made a study comprising four parts: (*a*) general technique in the preparation of the moist chamber; (*b*) the physical conditions—optical quality, capillary action, aseptic condition and moisture—in the chamber; (*c*) special techniques—fixation and coloration of the preparations, quantities of material, microcultures on gelatin or cellophane, and tissue culture; (*d*) using the chamber in micromanipulations for isolation of microorganisms.

Thaysen and Morris (1947) describe their extensive preparation of microtools for use with the micromanipulator. Because the tools required for the more delicate processes of micromanipulation are not commercially available, and existing equipment for their making is expensive, some cheaper equipment is described, comprising a gas forge and an electric forge, which together suffice to prepare the tools needed for isolating single cells, for injecting and dissecting living cells and tissues, and for determining their electrical potential. The microtools illustrated are microloop, microneedle, micropipette, microelectrode, microknife and microburner.

MICROINSTRUMENTS. Microinstruments when motivated by a mechanical hand (micromanipulator) can function in many ways, depending on the design of the instrument. While many microtools are made by hand, special machines have been designed for pulling or drawing micropipettes.

Richter (1947) invented a precision micropipette trimmer for perfecting the tips of micropipettes and microneedles for use with the Chambers' micromanipulator.

Hofmeister (1940) used the Schouten (1935) and Leitz-Du Bois

(1931) pipette pullers. For his micromanipulative work on diatoms he used a so-called Gleitmikromanipulator von Zeiss and the Chambers micromanipulator. He assembled a double instrument, employing two microneedles in two holders (left side, micropipette; right side, single microneedle) for micrurgical studies. For microinjection studies involving plant cells he again employed both micromanipulators. He studied onion scale slices and injected vacuoles with one-micron pipettes, using water as a head instead of oil. Cells were also injected with other chemicals and substances including sugar and calcium chloride.

Sheffield (1939) performed numerous microdissection, microinjection and microinoculation studies with plant viruses. She tested the cell contents, the stability of intracellular inclusions, the virus content of the cells. By means of a microcutting needle device, cells were divided into two parts with one containing the inclusion body. The inclusion body was then removed by exerting very gentle pressure at the base of the cell or by replacing the microcutting tool with a micropipette and removing the body by suction. Usually ten to twenty inclusion bodies were collected before diluting same for inoculation purposes.

Kassanis and Sheffield (1941) in their micrurgical studies reported variations in cytoplasmic inclusions induced by three strains of tobacco mosaic virus: spindle-shaped bodies, masses of short, needle-like fibers, extremely long coiled fibrous forms, and new amorphous forms. All these arose either directly or from preexisting inclusions of the previously recorded types.

Hildebrand (1941) in micrurgical studies demonstrated that more than 90 percent of crown gall bacteria (*Agrobacterium tumefaciens*) grew in sterile juice extract from young tomato plants. Single bacterial cells microinoculated into deep wounds on tomato stems produced galls in ten to 60 percent of the trials compared to ten percent or less in shallow wounds. When ten or more bacterial cells were injected, gall formation resulted in about 90 percent of the trials with the deep wounds compared to 20 percent or less with the shallow wounds. Thus it appears that depth or type of wound is more important than number of bacteria. Individual plant cells when injected with one or more bacterial cells always gave negative results. Living bacteria were recovered from only one of 20 plant cells as late as three weeks after inoculation. Minute wounds, produced by gently rubbing a polished needle with bacteria on stems and petioles, resulted in tiny galls close to the minute wounds associated with collapsed hair cells. Very few bacteria were observed in the tiny galls. A further elaboration of this study appeared the following year (Hildebrand, 1942).

Schure (1934) reported damaging action of substances dissolved from glass in single-cell cultures. This discovery was made in an effort to obtain pure cultures of *Reticularia lycoperdon*. The damaging substance suspected was probably silica.

In 1929 Whitaker (1929) described the construction of microthermocouples, small enough and strong enough to be inserted into small living cells or tissues. They are small enough to measure light absorption of a single plastid in a cell. The method is to draw various metals in glass to wires or filaments of exceedingly small size to make electrodes of less than one micron in diameter. Iron and platinum were found to work better than copper and constantan.

Burch (1939) revised a micrometer screw top fitted to the plunger of a tuberculin syringe so that small movements can be made with accuracy. For calibration the microsyringe is filled with mercury and the amounts ejected are weighed.

The Dean and Fletcher (1942) micrometer burette combines a micrometer with a syringe. The anvil of the micrometer is cut off and a glass syringe mounted on a simple clamp in line with the spindle. This instrument can be mounted and held by a universal burette clamp which can be attached to a ring stand. The micrometer burette can be calibrated to one part in 1000 independent of the speed of delivery. It can be dismounted and cleaned and oven-sterilized if necessary, permitting use of several solutions in the same burette. This would seem to be a superior instrument to the microburette recently described by Scholander (1942).

The Scholander (1942) microburette is a modification of the Rehberg (1925) burette. In it mercury is displaced by the spindle of a micrometerlike device. Moving an ordinary micrometer spindle through 25 mm. displaces around ¾ cc., which is precisely divided into 2500 parts with an accuracy of 0.1 mm. The solution is sucked into the bulb by turning the spindle, and the instrument is ready to use. The disadvantages of employing mercury are that the instrument gets dirty easily and can be used only with solutions that do not attack mercury.

Conger (1944) developed a simple method for micrometric ruling, and some applications. A crystal of silicon-carbide or carborundum, or a black diamond is mounted with sealing wax or dental impression compound on a micromanipulator or other rigid lever which is regulated by two sets of screws. This is attached to the front of the microscope with the crystal in the field of vision and serves as a cutting tool. The mechanical stage forms the ruling engine. As many as 30,000 lines per inch can be made without difficulty.

Wagner (1945) has developed an experimental microdrill for obtaining material for a microchemical test from some of the harder minerals. It consists of a motor mounted on a wooden base, a flexible shaft connected to the motor at one end and to a handpiece and chuck-drive arrangement at the other, and a chuck to hold the cutting tool.

The Arens (1939) apparatus determines the turgor pressure in a single cell with a manometer and has been used for measuring turgor pressure of large cells of *Nitella, Caulerpa* and *Valonia* without injury to the cell. All that is necessary is to place a light behind the window of the apparatus and project the bending of the cell on a screen, while the pressure can be read on an illuminated manometer.

Policard (1939) reported on a microincineration method for studies on mineral distribution in cells and tissues. If properly performed it gives a reasonably true representation of mineral distribution in living cells and organisms.

Schaede (1940) demonstrated root nodule bacteria in living gall cells.

Castle (1940) measured the rotary component of growth of a single plant cell, the sporangiophore of Phycomycetes, and observed significant differences in the increments in growth in successive 1/5 second intervals with the average change in growth rate between two and five percent of the average.

Chambers (1941) demonstrated that the so-called "blue nuclei" in tulip petals are the result of injury causing the pigment, which normally is restricted to the cell vacuole, to be preferentially absorbed by the nucleus when released into the cytoplasm by crushing injury.

Chambers and Black (1941) demonstrated the appearance of large numbers of phantom nuclei upon deplasmolysis of cells previously exposed to electrolytes, whereas vitreous nuclei appeared infrequently.

Lotze and Yiengot (1941) devised an apparatus for measuring microscopic objects, such as the marginal bodies occurring in the erythrocytes of infected cattle. The apparatus measures down to tenths of microns and consists of a microscope fitted with an oil-immersion lens, a 15 × ocular, and a camera lucida from which the mirror had been removed plus a light box two feet square and one foot deep fitted with four electric lights and placed about ten feet away from the microscope in a lateral direction. The open side of the box was turned toward the opening in the side of the camera lucida and was covered with a cardboard in which the scale had been made.

French and Anson (1941) demonstrated oxygen production by isolated illuminated chloroplasts suspended without much other cellular matter in sucrose solution with buffer, ferric oxalate and potassium ferricyanide. Spinach proved a good source of active chloroplasts. The reaction appears to involve catalysis by an enzyme, since it is stopped by warming, by Duponol, a protein denaturant, and by enzyme poisons. At low temperatures more O_2 is obtained, due to the high temperature coefficient (3.0) of inactivation of some constituent. The activity of a certain preparation of chloroplasts suspended in 0.5 M. sucrose had a half-life of 26 minutes at 35° C. An improved method of preparation of chloroplasts was carried out at a low temperature. Chloroplasts may be stored for a day or two and lose activity much more slowly in nitrogen than in air.

Gordon and Chambers (1941) studied the relation of particle size of a number of water-soluble acid dyes to their penetration into three types of living animal cells (tissues). Dyes with particle radii of 5.5 ± 0.4 to 6.4 ± 0.5 Å or less penetrated the ciliated epithelial cells. The upper limit for penetration of muscle fibers or bladder epithelium was 6.5 ± 0.4 to 7.3 ± 0.9 Å.

Seifriz and Epstein (1941) demonstrated shock anesthesia to protoplasm in their experiments employing one of the myxomycetes, *Physarum polycephalum.*

Guttenberg (1943) isolated plant cells of the stigma mucus of *Cymbidium lowianum* and cultured them in an attempt to secure evidence for the existence of hormones for cell proliferation. The substances tested (heteroauxin, thiamin, etc.) markedly stimulated the elongation of the cells, but cell division was not observed in any case. The enlargement induced was not typical of the proliferation process, and accordingly no specific substance has been tested which promotes cell proliferation. This report neglects to mention the wound hormone traumatin.

Moist Chambers and Special Chambers

Comandon and De Fonbrune (1938) devised a special moist chamber and discussed the advantages from its use for the study of living microorganisms, tissue cultures, and in micromanipulation, including single cell isolation, microdissection and microinjection.

Thaysen and Morris (1947) describe a simple hanging-drop micromanipulation chamber. The chamber was designed to accommodate the various microtools needed for isolating single cells, for injecting and dissecting living cells and tissue, and for determining their electric potential.

Kamiya (1940) placed small bits of *Physarum polycephalum* plasmodia on cover-glasses coated with agar and noted that they soon spread into thin sheets which later developed protoplasmic strands. One form developed that could be arranged so that there were two protoplasmic bodies connected by a single strand. Such a plasmodium was inverted over a chamber which was divided into two compartments, A and B, by an agar block. The construction is such that the two compartments may be kept air-tight without the wall separating them blocking protoplasmic flow in the connecting strand. One of the two compartments is kept at constant atmospheric pressure, whereas the pressure in the other compartment is under control. Protoplasmic movement goes on normally when pressure is same. Difference in pressure alters flow—reduced pressure accelerates flow; increased pressure retards flow. Removal of pressure causes normal flow again.

Thomas (1941) described a technique for continuous microscopic observation. The cavity of a depression slide was filled with an excess of freshly heated (sterile) oil. A small portion of a semi-liquid culture of the organism to be studied was then transferred by pipette from bottom of culture tube to the center of a sterilized coverslip. The coverslip is inverted over the depression of the objective slide in a manner that will eliminate all bubbles of air. The excess oil will be displaced to form a perfect seal. The microscope should not be disturbed because in a prolonged observation of a specific germinating spore or a dividing cell, any movement of the microscope may cause the organism to be carried from the field. By changing the distance between bulb and stage or by varying the intensity of bulbs, one can obtain the temperatures desired.

Brown (1942) developed a method for the study of the development of fungi (*Aspergillus niger* and *Monilia* spp.) from single spores or hyphal fragments. It involves a special glass slide (76 × 28 × 2 mm.), the center of which is occupied by a polished, slightly depressed, concave table chamber, 1 cm. in diameter, surrounded by two concentric moats connected by a short communicating moat and providing for an air inlet. A square 25 mm. cover glass is held in place by mineral oil contacting six points on the slide after a full, large (4 mm.) loop of inoculated culture medium (Sabouraud's agar) has been placed at the top of the central table. Contamination and desiccation are obviated by restricting air to that entering through the moats out of contact with the culture medium.

Weston (1942) made a Petri dish holder for mechanical stages which firmly clasps the dish and carries it by a frame that fits snugly into the slide holder of the mechanical stage. A further modification was a device for holding the Petri dish top over the bottom to permit mechanical manipulation of needles and pipettes in the dish.

Horner (1945) designed three stage incubators to be used in microscopic study. These instruments afford the study of precisely predetermined volumes of culture media under the microscope, with closely controlled conditions affecting development of the culture in respect to variable temperature, variable electromagnetic or electrostatic field, or a combination of these or different conditions. It is often desirable to be able to determine accurately the rate of propagation. This may be accomplished by accurate counting from time to time of the number of cells present in a precisely determined volume of the medium. It is desirable to have quantities sufficiently small for counting directly, or by dilution, in a known volume, and to permit counting of subsequent divisions of the cells in a given fraction of the original volume. The stage incubators are constructed of a non-corrosive material which may readily be cleansed and sterilized at high temperature with or without the use of a disinfectant.

Richter (1948) invented an improved moist chamber slide for use in micromanipulation. The method of keeping the chamber moist precludes chance disarrangement of manipulation needles while in use and provides a measurable control of relative humidity.

Light Source or Mechanism

Undoubtedly the finest lamp for micrurgical studies is the new so-called "Universal lamp" put out by Spencer Lens Division of the American Optical Co. at Buffalo, New York. By means of a transformer and rheostat the light intensity can be varied at will. A distinctive feature is that the lamp does not heat up and therefore has no operation hazard from burning on contact. Because of the low and steady heat component in its light rays, one can operate the instrument for one or two hours without the condensation droplets in the moist chamber being destroyed by enlargement and running together.

Melezer and Venkel-Wlassics (1939) suggest the use of a quicksilver vapor lamp as a light source for fluorescence microscopy and microphotography. Their lamp has a wave length between 310 and 390 millimicrons and operates on 220 volts A.C. This lamp was developed by the Simons Company and is suggested for use in place of the expensive low-voltage ultraviolet arc lamp using iron electrodes. This lamp is designed on the general plan of the ordinary tungsten incandescent lamp with Edison base, and burns evenly, emitting a constant source of light.

THE BURCH REFLECTING MICROSCOPE

The birth of the science of bacteriology was made possible by the invention of the light microscope. This instrument, however, even after being perfected, has limitations, and the recent invention of the super- or electron microscope, while assisting in the problem, has not completely filled the long felt needs within the range of light microscopic objects. The invention of the reflecting microscope by Burch (1947?) certainly promises a significant advance in this direction.

While the idea of microscopes using mirrors, similar to those of astronomers, instead of lenses is not new, the most significant development along this line is the Burch reflecting microscope, as discussed by Barer (1947). Barer states that its two unique features are that it is completely achromatic and that it has an unusually long working distance. It is ideally adapted for absorption spectroscopy of cellular constituents. Whereas the quartz microscope is suitable only for plotting an absorption curve over a very narrow band of wave lengths, with the reflecting microscope the entire absorption from the far ultraviolet to the infrared can be plotted on the one instrument. The quartz microscope cannot be used below 1900 Å, since quartz becomes opaque in this region. The fluorite lenses could be used down to about 1300 Å, but fluorite is very expensive and such lenses are difficult to make. Below 1300 Å no substances suitable for making lenses are known. It seems reasonable to suppose that the reflecting microscope could be used down to 1000 Å, at least, and probably even lower. If the immense difficulties can be surmounted one day it may be possible to use wave lengths approaching 100 Å, and this would provide an instrument with a resolving power of the same order as the present electron microscope.

In the present reflecting microscope, when in focus the distance between the mounting of the small mirror and the object under observation is 13 mm. This distance could be increased to 20 mm. if the mountings were redesigned. For various types of micrurgical work, and especially for micromanipulation, long working distances are a great advantage particularly because they enable a microinstrument to be manipulated between the cells observed and the small mirror.

According to Barer (1947), ultraviolet microscopy should be extremely important in biology for two reasons. In the first place, the shorter the wave length of light, the higher the resolving power and useful magnification of the instrument. Thus ultraviolet light of wave length 2500 Å should give about twice the magnification obtained with green light of wave length 5460 Å. Secondly, certain constituents of the living cell selectively absorb some of the ultraviolet wave lengths with the cell nucleus showing a strong absorption in the region of 2500–2700 Å. Unstained living cells photographed in light of such wave length will therefore appear as if the nuclei have been stained. Here we have the great advantage of the reflecting microscope. To take a photograph by ultraviolet light, one focuses the image by visible light, changes to the ultraviolet source, and takes a photograph without change of focus. The same thing also holds for infrared light. Thus ultraviolet light of any wave length can be used with the reflecting microscope, which is not true for the quartz microscope, essentially a monochromatic instrument, that corrects for only one wave length.

Barnard (1939–1940) studied the value of ultraviolet microscopy for the investigation of the smallest living things—small bacteria and viruses. He clearly delineates the fact that the ultraviolet light technique—observing a visible dark-ground image and determining the size of observed bodies in terms of their brightness—is apparently little appreciated by microscopists. The same method is well known and widely used in astronomy. The ultraviolet light technique was used for photographing eight species of bacteria at magnifications between × 2000 and × 3200, and seven species of animal virus all at the × 2250 magnification. Some of the viruses appeared similar to some of the larger recognized microorganisms. None of the viruses appeared similar to ordinary bacilli. The resemblance of the viruses to cocci was much closer. The vaccinia virus departs from the spherical shape only at subdivision, behaving at that time like micrococci. When the ultraviolet light print showed dense opaque masses, the virus particles apparently had a size of less that 50 $\mu\mu$.

Micrurgical science should benefit tremendously when more of these instruments become available.

GERM-FREE CULTURE OF ANIMALS

Glaser and Coria (1935) described a simple method for preparing yeast extracts useful for culturing certain protozoa. *Paramecium multimicronucleatum* was purified and cultured on a special medium. Cultural differences between these species and *P. caudatum* were noted. Three ciliate strains from thermal waters were also purified and cultivated. This work demonstrated that protozoa could be freed from other living microorganisms and that they yielded biological reactions that may prove useful in the separation of morphologically similar forms.

Glaser (1943) points out that in the germ-free culture of invertebrates it will be necessary to recognize that in nature, after species identification and genetic purifications, every animal is still a combined zoological and botanical garden. By special reliable techniques he was able to grow certain invertebrate animals (protozoa, nematodes, insects) germ-free. Can one imagine a cultural growth in a test tube containing millions of teeming nematodes in pure culture?

Hoepple, Feng and Chu (1938) attempted to culture nine species of helminths of vertebrates in artificial media. Isotonic salt solutions gave the best results for intestinal helminths. Serum, undiluted or diluted with or without various substances, gave good results for bile-duct, blood and tissue parasites. Transfer to fresh media at intervals is necessary for keeping parasitic stages alive over a long period. The longest survival period was about five months for *Chlorchis sinensis*.

Johnson and Baker (1942) developed a successful culture medium for growing *Paramecium multimicronucleata* germ-free, employing pressed yeast juice sterilized by passage through a Seitz bacteriological filter.

Jackson (1945) developed a method for observing and staining live insect blood under oil immersion. The procedure consists essentially of immobilizing a larva beneath a coverglass in such a position that one antenna is pressed against the glass, cutting off the end of the antenna, and observing the blood as it pulses forth against the glass.

SINGLE CELL ISOLATION BY THE TREXLER AGAR-COVERSLIP METHOD[3]

1. Apparatus. Reyniers (1938) micromanipulator and moist chamber; special agar freed from debris; ring slides; coverslips

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[3] The method here described was demonstrated by Philip C. Trexler on November 14, 1940, before the Advanced Plant Pathology 201 class at Cornell University, Ithaca, New York.

(¾″ square and 0 thickness); shellac, paraffin, porcelain, vaseline; microneedles; micropipettes.

2. Clearing of agar. Autoclave Difco Brain Heart Infusion agar (1.5%) in centrifuge tubes of about 15 cc. capacity. After sterilization the tubes are placed in a heated metal cup with a water cushion. Balance and heat the cups with the tubed agar to about the boiling point. Centrifuge at high speed for about 15 minutes until agar is solidified. Hold the tube in a strong cross light; if it looks clear it should be satisfactory for use. Other methods of clearing agar (centrifuging, white of egg, etc.) can be used instead. From experience he found that by heating in the Arnold steamer, most particles usually settle out in about one-half hour.

3. Preparation of ring slides. Materials tested include varnish, paraffin, porcelain cement and brass rings. Porcelain cement has definite advantages. Insolute porcelain cement comes in bottles in powder or paste form. With a toothpick or other device several porcelain (or paraffin, etc.) ring slides are prepared to act as chambers for holding the coverslips. The ring on the "ring" slides should be about 1/32 inch high and somewhat less in diameter than the coverslip. More recently brass rings have replaced all the rest because it is easy to keep their surface parallel to the slide. Trexler preferred ring slides to depression slides.

4. Preparation of Coverslips.

(*a*) Check "ring" slides. If uneven, polish surface smooth with a stone. Examine for depth and diameter correctness.

(*b*) Prepare a bulb pipette, using a 1 cc. rubber bulb and a piece of glass tubing drawn out to a fine tip.

(*c*) Clean the coverslip (Preference is for ¾ inch square glass of No. 0 thickness) by dipping in alcohol or acid-alcohol followed by alcohol. Then polish with lens paper. Hold coverslip in a pair of forceps.

(*d*) Carefully heat a tube of agar over a Bunsen flame. Apply the heat near the top of the agar column only, so that perhaps the upper half inch becomes melted.

(*e*) Flame the coverslip in order to sterilize and heat it above the temperature of agar solidification.

(*f*) Likewise flame the pipette for the same purpose.

(*g*) Suck up about ½ cc. agar in the pipette and place a drop on the warm coverslip. Turn the coverslip at an angle so that the fluid flows to one side of the drop and remove as much as possible with the pipette. Immediately set the coverslip on the ring slide. Press firmly on the vaseline seal so that a tight connection is made.

5. Procedure.

(*a*) A coverslip is inoculated with a young culture of the organism being studied.

(*b*) The inoculated coverslip is placed on the moist chamber located on the microscope platform.

(*c*) Single cells are isolated by the Dickinson (1926) method.

(*d*) The locations of from six to 12 well isolated cells are marked on each coverslip by tearing the agar film with the microneedle.

(*e*) The agar coverslip is then transferred to the "ring" slide for incubation.

(*f*) When microcolonies develop, the agar coverslip is replaced on the moist chamber.

(*g*) Microcolonies are transferred to culture.

6. The Trexler modification of the Reyniers (1932) micropipette puller.

(*a*) The principal modification was to pull micropipettes with a bent tip.

7. A Tungsten (Pfansteel) microneedle for use in micrurgy. The idea is to polish a tungsten wire to a very fine point by electrolysis, using the principle of "atomic polishing".

(*a*) A battery jar arrangement is set up with the jar containing 1 M. NaOH and a reducing agent such as sodium hyposulfite.

(*b*) Two electrodes of tungsten were installed in the improvised cell and held by battery clamps.

(*c*) A transformer (15 volts or less) is connected. Either A.C. or D.C. may be used.

(*d*) If the wires are dirty they can be cleaned in potassium dichromate solution.

(*e*) When employing a 15-volt transformer, a fine tungsten tip is formed in about two minutes.

(*f*) The depth of the tungsten wire in the NaOH solution and the amount of metal removed determine the taper.

(*g*) The tungsten microneedle can be used for many purposes, especially for transfer of microcolonies. The great advantage of this microneedle is that it can be flame sterilized and used again and again.

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GROWTH OF FUNGI IN SYNTHETIC NUTRIENT SOLUTIONS. II.[1]

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INTRODUCTION

The years that have passed since publication of the previous review (108) have covered a period of expanded interest in the nutrition of fungi. The previous investigations on the organic and mineral needs of fungi had laid a firm foundation for these more recent studies. Investigations were barely under way at that time on the genetic factors concerned in the erratic behavior and "degeneration" of fungi (mutation) in culture. A fresh impetus too was given by studies of antibiotics and fungal decay of materials, since little was known of the specific nutritional requirements of the fungi dealt with, or of their capabilities.

While it is true, therefore, that progress has been made, it is also a fact that a great deal still remains to be done. Some fungi, such as the rusts, the powdery and the downy mildews, cannot be cultured on any media. Other important problems awaiting solution are, to mention but a few, the relation between constitution and assimilability of the hexoses and the chemical reactions of their transformation into amino acids. Though fungi offer excellent material for studies on mineral deficiencies at fixed carbohydrate levels, no investigators have concerned themselves with their relation to protein-carbohydrate metabolism. The importance of resistance and pathogenicity in fungous diseases has not as yet led to any intensive studies of the products of fungal exosmosis in culture from this viewpoint. These products probably play an important part in these phenomena.

Reviews of various phases of investigation bearing on nutritional studies have appeared from time to time during the interim. Schopfer (106) and more recently Robbins and Kavanagh (90) have reviewed what is known on the vitamin requirements of fungi. Perlman (84) has summarized the data on micronutrient requirements, though primarily with relation to the products of fermentation and not growth. An excellent summary on mutations and variations in fungi pathogenic to plants has been published by Christensen, Stakman and Christensen (19) and in *Neurospora* by Beadle (4). Microbial genetics has also been reviewed by Lindegren (64).

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The subject matter of this review includes the nutritional requirements of fungi for carbon and nitrogen compounds, minerals and vitamins for growth and reproduction. A brief section on mutation in fungi has also been included as in the previous review and for the same reason—a variable organism cannot be used to obtain reliable and constant results in nutritional or pathological studies. The short section on pathogenicity has been added in the hope that it may induce investigations in synthetic solution of the products of exosmosis of fungi, and of the influence of nutrition thereon.

This review makes no pretense at completeness of coverage. Many probably important articles could not be obtained, whereas others were in languages unfamiliar to the writer. In an attempt to conserve space, the names of the fungi are given without mention of taxonomic authorities, even though stated by the author. The citations, lastly, are not mentioned in chronological order of publication but usually in an alphabetical arrangement according to the author's name.

CARBON REQUIREMENTS

Additional information on carbon utilization is largely confined to the extension of previous results to additional species. The data are usually on the basis of compound percentage content. Comparative results on carbon utilization would be of still greater value if equal carbon or energy levels, or molecular weights were used. An insight might so be had into the chemical factors concerned with carbon metabolism. It is also important to remember that results with mixtures of carbon compounds are not directly comparable to those with single compounds. Mixtures may be metabolized more readily in some instances than are single compounds. Or a compound practically unusable may be readily employed on admixture with another.

Most fungi grow readily with sucrose and the hexoses—D-glucose, D-mannose, D-fructose—, and in addition the pentoses

D-xylose and D-arabinose to a lesser degree. A comparative study of growth of *Aspergillus niger* with a large number of carbon compounds has been made at the same carbon level (112). The yields in grams per gram of carbon supplied were: D-glucose, 1.43; D-mannose, 1.46; D-fructose, 1.38; L-sorbose, 1.26; and D-xylose, 1.09. Other compounds gave much poorer growth. Enolization was not a factor in sugar utilization. All pentoses and hexoses having an L-3 carbon atom and a D-4-carbon atom were assimilable except the epimers of D-xylose and probably L-sorbose. These configurations are identical with those of the alpha and beta carbon atoms of the natural β-hydroxy amino acids.

Apparent exceptions are numerous, however, to the above generalizations. D-mannose could not be used by *Achlya* sp., *Isoachlya anisospora, Saprolegnia delica;* nor sucrose by *I. anisospora* and *S. monoica* (8). *Coprinus lagopus* is reported to require cellulose and to be unable to use hexoses (128). The majority of fungi respond approximately as does *A. niger: Phymatotrichum omnivorum* (9), *Rhizopus suinus* (21), *Stereum gausapatum* (46), *Venturia inaequalis* (57), and *Allomyces Kniepsii, Blastocladiella variabilis, Rhizophilyctus* sp. (86). *Leptomitus lacteus* (105) cannot use sugars.

The problem of preventing molding of fabrics has led to increased study of cellulose decomposition capacities of the fungi. Extensive surveys of large numbers of fungi have been published (54, 71, 131). The effect of nitrogen nutrition on cellulose decomposition of *Trichoderma viride* (10) and of *Coprinus lagopus* (128) has also been studied to some extent.

The utilization of amino acid carbon at an identical level has been studied with *Aspergillus niger* (111). The highest utilization factors obtained were L-proline, 0.22; L-aspartic acid, 0.21; L-glutamic acid, 0.23; L-ornithine, 0.10; and L-arginine, 0.10. A mixture of L-proline, L-glutamic acid and L-ornithine had a carbon utilization factor of 0.82 as compared to that of 1.17 for sucrose. The utilization of carbon in this mixture was not improved by addition of DL-alanine, L-aspartic acid, glycine or L-hydroxyproline. It appears probable therefore that this fungus is capable of using a mixture of its amino acids previously formed from sugar almost as efficiently as hexose in the elaboration of the many compounds needed for its growth and development. Some evidence was obtained, however, that substitution of the hydroxy for the amino acids was still more effective (112) in supporting growth at highest efficiency. Roine (97*a*) reached a similar conclusion on the basis of chemical studies with yeast, concerning primacy of dicarboxylic amino acids, particularly glutamic, in amino acid syntheses from ammonium nitrogen. Results and conclusions almost identical with those published for *A. niger* were recently reported for yeasts (106*a*) also.

Similar investigations have been made with *Fusarium oxysporum* var. *lycopersici* and *Penicillium roquefortii* (39). Of the 25 compounds tested, 17 permitted growth with amino acid carbon in 0.5% concentration. Growth was estimated visually. *Aspergillus oryzae* (125) was grown on amino acids as sole source of both carbon and nitrogen. Yields were reported to rank as follows: isoleucine > alanine > glutamic acid > arginine, phenylalanine > histidine > tryptophane, valine, proline > glycine, serine, aspartic acid > lysine. Respiration ranked in this order: alanine > isoleucine, phenylalanine, tryptophane > leucine, serine, valine > proline, histidine, glycine > arginine, glutamic acid > aspartic acid > lysine. It is evident that growth and respiration are not identically parallel phenomena as concerns amino acids at least.

The re-elaboration of respiratory carbon dioxide by *Aspergillus niger* was again demonstrated and its inhibition found to enhance the effects of micronutrient deficiencies (110). The conclusion was reached with *Rhizopus nigricans* in shake cultures that carbon dioxide utilization by fungi was less than that attained by propionic acid bacteria (25). Radioactive carbon of carbon dioxide was largely localized in the acid-insoluble material of the fungus and in the carboxyl groups of formic and citric acids.

Respiratory studies with carbon compounds have also been made with *Penicillium notatum* (132) and *Oospora lactis* (136). Rate of oxidation in the latter organism (glucose > xylose > arabinose) appeared to parallel yields in *Oospora*. The data for *Penicillium* showed several divergencies, however, in that galactose was as readily oxidized as glucose or mannose, while sucrose, fructose and xylose were no better than arabinose. The accumulation of yield and respiration data for a large number of compounds and fungi would appear to be most desirable for an understanding of carbon metabolism.

Failure to grow on diose, triose, etc. sugars has usually been interpreted as signifying that the fungus was lacking in sucrase, maltase, lactase, diastase and so on. Estienne, Castagne and Bertrand (24) examined a large number of fungi for ability to grow on lactose and tested the powdered fungi for lactase. They reported that high yields on lactose were not paralleled by high lactase content of the organism. They found strain differences to exist and little or no lactase in the higher fungi.

A brief article by Caputto *et al* (18) on galactose utilization by *Saccharomyces fragilis* is of special interest to the writer. Yeasts can be "trained" to ferment this sugar. Lindegren (65) has found that they may also be bred for ability to employ galactose. It appears now that *S. fragilis* produces galactokinase which catalyzes transphosphorylation between adenosine triphosphate and galactose. The reaction product, galactose-1-phosphate, was transformed to glucose-6-phosphate. Apparently the hexose-amino acid conversion reaction in yeasts using galactose is probably the same as in non-galactose using strains.

NITROGEN REQUIREMENTS

The generalization that fungi capable of using nitrate-nitrogen can also use ammonium-nitrogen (108) appears not to be without apparent exceptions. *Alternaria solani* (78) does not grow well on ammonium-nitrogen as compared to nitrate-nitrogen. On the other hand, of 14 species of *Marasmius* all could use ammonium-nitrogen and only one nitrate-nitrogen also (61). A few other fungi reported to thrive on ammonium-nitrogen include *Achlya* sp., *Isoachlya anisospora* var. *indica, Saprolegnia delica, S. monoica* and *Brevilegnia gracilis* (7). Only the last could also use nitrate. *Ophiobolus graminis* can utilize nitrate (35). Ammonium is used by *Collybia dryophila* (62), *Cenococcum graniforme* (73) and yeast (79, 107).

Good growth has been reported in synthetic media supplied with inorganic nitrogen by *Trichoderma viride* (10), *Sclerotinia sclerotiorum* and *Alternaria solani* (78), *Saccharomyces cerevisiae* (80), *Phacidium infestans* (81), *Memnoniella echinata* and *Stachybotrys atra* (82), *Sclerotinium delphinii* (83), *Pythium arrhenomanes, P. deliense, P. graminicolum* and *P. mamillatum* (99), *Saprolegnia delica* (102), *Aspergillus oryzae* (125) and *Phycomyces blakesleeanus* (12).

Asparagin was a good source of nitrogen for *Leptomitus lacteus* and *Apodachlya brachynema* in the presence of acetate, but neither could use inorganic nitrogen (104), and for *Penicillium digitatum* (134). Forms growing poorly or not at all on inorganic nitrogen included *Blastocladia Pringsheimii* (17), *Lactarius deliciosus* (74) and *Pythium indigoferae* (101). A mixture of amino acids could not replace peptone for the last organism. A large number of fungi causing decay of living oaks were successfully cultured on malt agar (22), but no attempt to use synthetic media was made.

Growth responses of fungi to amino acids fall into two categories, specific and non-specific. *Blastocladia Pringsheimii* (17) requires methionine but is aided in growth by supplementation of its solution with still other amino acids. Amino acids increased the growth of *Cenococcum graniforme* when grown on ammonium-nitrogen (73), and of *Lactarius deliciosus* (74).

The amino acid compositions of *Saccharomyces cerevisiae, Rhodotorula rubra, Rhizopus nigricans, Aspergillus niger* and *Penicillium notatum* have been determined by Stokes and Gunness (124). Histidine, arginine, lysine, leucine, isoleucine, valine, methionine, threonine, phenylalanine and tryptophane were estimated. The authors concluded that the amino acid compositions are qualitatively and quantitatively a stable and characteristic property of the cell under fixed conditions of growth. The fungal mycelia contained 50% more protein prior to sporulation than after sporulation. The individual amino acid content was found to vary with environmental factors, such as the medium, aeration; and with age.

Quantitative studies of amino acid utilization must be interpreted on the basis of type of nitrogen assimilation employed by the fungus. One that is unable to use inorganic nitrogen may be assumed to employ the amino acids as such. Another that can use inorganic nitrogen may make use only of ammonium-nitrogen subsequently to deaminizations. *Aspergillus niger* and the yeasts fall into the latter group.

A quantitative study of the growth of *A. niger* with amino acids (111) disclosed that these fell into three groups. Nitrogen in alanine, arginine, aspartic acid, glutamic acid, glycine, hydroxyproline, ornithine and proline proved fully equivalent to inorganic nitrogen. Serine, threonine and tryptophane gave yields approximating 50% nitrogen assimilation. The balance of the amino acids gave very poor growth. It was pointed out that none of the first group contained a stable cyclic group or branched carbon chain, nor were they among those incapable of synthesis by the rat. It was further pointed out that none of the amino acids except three of the eight in the first group could also be used as a source of carbon supply by the fungus. Glutamic acid, ornithine and proline are considered for these and other reasons to be precursors of other amino acids.

Nielsen (79) in similar experiments with yeast found practically complete utilization of amino nitrogen in the same eight amino acids and in leucine and tyrosine also. The reason for failure to obtain complete agreement with the results when using *A. niger* may be associated with the use of beer-wort instead of the pure vitamins. Schultz and Pomper (107) studied utilization of amino-nitrogen by 19 species in six genera of yeasts. Excellent growth was obtained with five of the above eight amino acids (hydroxyproline, ornithine were omitted) in 16 or 17 of the yeast species. Glycine gave good growth with only five species, and tyrosine with seven species. Leucine nitrogen was effective with 16 species.

Growth and respiration of *Leptomitus lacteus* (104) and respiration of *Penicillium chrysogenum* (133) with amino acids have also been investigated. The results showed both similarities and differences to the growth data obtained with *Aspergillus niger*.

An interesting observation by Robbins and McVeigh (97) is that *Trichophyton mentagrophytes* is quite sensitive to 50 p.p.m. of L-hydroxyproline. This amino acid gave a similar response with *T. gypseum, T. purpureum, Epidermophyton flocculosum* and *Microsporum canis.* Nineteen other species were not inhibited in growth under these conditions. In the writer's view these results afford an interesting demonstration of contrasting action of amino acids in the free as compared to the bound condition in proteins. This response to L-hydroxyproline is also given by tobacco seedlings which are killed at a concentration of five p.p.m. (116).

MINERAL REQUIREMENTS

Studies of the mineral nutrition of fungi have progressed relatively slowly since the previous review in this journal. Perlman (84) has recently reviewed the new developments with respect to micronutrients but has concerned himself to a great extent with the metabolic products of fermentation. Since such studies have as their object the maximum production of a metabolite, adjustment of the components of the nutrient solution are in general opposed to optimum growth (26). It is not improbable that the unbalanced nutrient solutions employed in fermentation may at times lead to a distorted and erroneous concept of normal metabolism.

The writer has published a brief summary of investigations on mineral nutrition with *Aspergillus niger* (113). Emphasis was placed on the fact that the quantity of each chemical element required for maximum growth was exactly proportional to the quantity of sugar in the medium. The minimum quantity of macronutrient salts per gram of yield was determined to be 100 mg. or 10.6% of the sugar present. The solution contained ammonium nitrate, potassium phosphate and magnesium sulfate and was adjusted to exact quantity with potassium nitrate and ammonium sulfate. Micronutrients were added in tested optimum quantities: iron, zinc, copper, manganese, molybdenum and gallium. Each element was present in minimal amount corresponding to individual determinations of its optimum for growth (114).

The specificity of potassium and magnesium for nutrition of *A. niger* (115) was checked by substituting lithium, sodium, rubidium and cesium for potassium, and beryllium, calcium, strontium and barium for magnesium. Only sodium and beryllium were even slightly effective and then only when potassium or magnesium, respectively, were 50% or more deficient. *A. niger* could assimilate sulfate, sulfite and sulfoxylic acid equally efficiently but could not use sulfide or disulfide. Alkyl sulfonates and sulfinates were equally effective as were cystine and methionine sulfur (109).

Laborey (55) determined the magnesium/phosphorus absorption ratio and concluded it to be a constant. The Mitscherlich equation was found to give good agreement with yields for magnesium, phosphorus and potassium. Magnesium absorption agreed with the formula $KX^{1/P}$ where K was a constant, $^{1/P}$ a constant less than one, and X the concentration of magnesium per 100 ml.

Most investigators have limited themselves in their work to the macronutrient elements enumerated under *A. niger*. The relatively few studies dealing with the known micronutrients included work with a yeast (53) found to require iron and zinc; *Phycomyces blakesleeanus* (58, 59); some of 14 species studied in the genus *Marasmius* were concluded to require zinc, manganese and calcium (61); *A. niger* could not be demonstrated to need manganese under other than acid conditions (68); the necessity of molybdenum for *A. niger* was again verified (76). *Phythiomorpha gonapodyides* and *A. niger* required more zinc and manganese than *Phycomyces blakesleeanus* (88).

A few investigations dealt with elements not hitherto demonstrated to be essential for any of the fungi. In a series of carefully executed experiments *A. niger* was found to increase 20.9% in yield through addition of vanadium (5). Vanadium was present in all fungi examined (6) in 0.15 to 156 p.p.m. A comparison of the need for calcium by various fungi was made in quartzware with spectroscopically pure chemicals and led to the conclusion that certain fungi required two to four p. p.m. of calcium in their nutrition (118). *Aspergillus niger* and *Fusarium oxysporum* were not diminished in yield by omission of calcium. *Rhizoctonia solani, Sclerotium rolfsii, Cercospora nicotianae, Pythium irregulare* and *Thielaviopsis basicola* decreased in yield when calcium was not added. *Rhizoctonia* decreased 85.7% in yield on omission of calcium, nor could any of a series of trace elements replace its action. Boron, zinc and manganese were found necessary for growth of *Fusarium vasinfectum, F. udum* and *F. moniliforme* when using the dithizone technique (135).

The not too distant future should provide information concerning the need of additional biologically essential elements by fungi. Too little use has been made of the calcium carbonate method for removal of trace element impurities and of the dithizone reaction for confirmation of their absence in the reagents. A paper on the preparation of chemicals free of trace element impurities for use as spectroscopic standards (52) verifies the biological data obtained with the carbonate method. A tube method of measuring growth in trace element and vitamin studies has been proposed (98). A new simple technique for preparation of silica gel media (126) from a commercial colloidal silica preparation should facilitate the study of fungi requiring solid media. No reports are as yet available concerning the application of 8-hydroxyquinoline for removal of molybdenum (47) in fungal nutrition studies. Additional light is thrown on the difficulty of freeing nutrient media containing amino acids from trace elements by a recent article (77).

The presence of cobalt in vitamin B_{12} and the probability of its synthesis only by bacteria would indicate the desirability of reaching higher levels of purity in micronutrient studies with fungi. A wide variety of forms having different requirements may be found to have specialized needs for trace elements. Those which the writer has studied, however, include only *A. niger* and fungal pathogens of the tobacco plant, and were found to have much the same needs for iron, zinc, copper, manganese and molybdenum.

VITAMIN REQUIREMENTS

A large number of papers have appeared in the past decade on the vitamin and other accessory compounds required by fungi. Details of these studies can be obtained in a book by Schopfer (106) and an excellent review by Robbins and Kavanagh (90). The earlier work has been amply verified and extended to demonstrate that vitamin and similar deficiencies are of very frequent occurrence among fungi. The fact that many of those successfully cultured grow very slowly would indicate, however, that many factors of this nature are still unidentified.

The compounds most frequently deficient in culture are thiamin, biotin and pyridoxine, though inositol and hypoxanthine are also needed in some cases. Yeasts (14) and presumably other fungi may require pantothenic acid and nicotinic acid. Vitamins A, D, E and K, riboflavin, ascorbic acid, folic acid and B_{12} which contains cobalt have not been reported as indispensable for any of the fungi. The data of metabolic deficiencies in mutant strains produced by chemical, ultra-violet and X-ray induction would indicate the possible occurrence of many other organic deficiencies. These include among other substances amino acids, purines, pyrimidines, nucleotides and choline.

No attempt will be made to list the vitamin requirements of all fungi reported upon. Many of the forms mentioned in the sections on carbon and nitrogen requirements cannot grow in synthetic media without vitamins. Only a few of the more recent publications are here included.

Sclerotinia camelliae (3) was found to require biotin, thiamin and inositol; *Graphium ulmi* (13), pyridoxine; *Schizosaccharo-*

myces pombe (20), biotin, nicotinic acid, calcium pantothenate, inositol and thiamin; *Melanospora damnosa* (38), biotin and thiamin; *Collybia dryophila* (62), thiamin and biotin; *Memnoniella* sp. and *Stachybotrys* sp., biotin (70); *Fusarium avenaceum,* biotin (91); *Phycomyces blakesleeanus,* thiamin and hypoxanthine (89); *Ceratostomella ulmi,* pyridoxine (93); *Trichophyton discoides,* pyridoxine, inositol, thiamin and as yet unknown compounds (96); *Ophiostoma catonianum, Ceratostomella ips, C. microspora, C. montium, C. annulata* and *C. ulmi* all required pyridoxine (94, 95); *Pythium arrhenomanes, P. deliense, P. graminicolum, P. hyphalostricton* and *P. mamillatum* required thiamin (100); and *Ophiobolus graminis,* thiamin and biotin (130).

Nine species of *Ophiostomella* (*Ceratostomella*) were investigated by Fries (29). *O. piceae, O. stenoceras, O. coeruleum, O. quercus, O. pini* and *O. multiannulatum* needed thiamin. *O. pini* also required biotin, and *O. multiannulatum,* pyridoxine. Only pyridoxine was indispensable for *O. ulmi, O. fagi* and *O. filiferum. Mitrula paludosa* needed biotin and thiamin with nitrogen supplied as ammonium salt, but good growth was attained without biotin on nitrates. A series of eight *Coprinus* species required thiamin, usually only the pyrimidine moiety sufficing (27).

Litter-decomposing Hymenomycetes (34 species, 13 genera) were found usually to require thiamin (63), while need of other substances was rare on glucose and ammonium-nitrogen agar. Mycorrhizal (tree) fungi frequently required thiamin and biotin, but no uniformity was found to exist (75). A group of ten species of *Ceratostomella* were found to suffer from partial to complete deficiencies of biotin, thiamin and pyridoxine (92).

Torulopsis utilis synthesized more thiamin, riboflavin, nicotinic acid and pyridoxine in cultures deficient in iron; and less biotin, inositol and p-aminobenzoic acid (60). Pantothenic acid synthesis was not affected, according to Lewis. Meissel (72) found thiamin injurious to *Endomyces magnusii* in the absence of biotin.

REPRODUCTION

It is almost traditional in botany to consider growth and reproduction as two opposing phenomena and that one must predominate at the expense of the other. Little mention is made in this connection, however, of everblooming plants in which both processes continue side by side. In the writer's opinion the major bases for this belief are those cases in which supplies of metabolites are insufficient for both processes. A contributing phenomenon is the fruiting of fungi when transferred to water or dilute solutions—but only probably when excessive quantities of nutrients have been employed in the medium. As concerns homothallic fungi grown in synthetic solutions, reproduction should be viewed as a normal and inevitable process consequent on normal preliminary growth.

The work of Coons, in which emphasis was placed on the use of dilute solutions as culture media, has been given a theoretical basis by certain observations made by Hawker (43). Optimum sugar concentrations for perithecia formation in *Melanospora destruens* (42) were lower than for growth, and increased growth substances increased the sugar optimum for perithecia formation. Both scarcity and excess of nutrient supplies may therefore prevent reproduction and presumably within limits fall within those for growth.

Improved sporulation because of added vitamins has frequently been observed. Thiamin was effective with *Ceratostomella fimbriata* (2) and biotin with *Sordaria fimicola* (1) and *Chaetomium globosum* (15) in perithecia formation. Biotin increased conidial production in *Memnoniella echinata* (15). Both biotin and thiamin were needed for formation of perithecia in *Melanospora damnosa* (38). Hexose phosphates increased perithecia in *Melanospora destruens* (44); thiamin that of *Pellia epiphylla* (69); and biotin, thiamin and pyridoxine for some of eight species of *Ceratostomella* investigated (92). Other studies on the nutritional factors of reproduction include those by Butler, Robbins and Dodge (16), Hawker (41), Hawker and Chaudhuri (45), Leonian and Lilly (59), Richards (87), Robbins and Ma (94), Voderberg (128) and Westergaard (129).

Too little attention has been paid to the possible influence of high solar light intensities on reproduction. Many fungi causing leaf diseases grow and fruit normally in bright sunlight. *Diaporthe phaseoli* from soybean (127) formed an increased number of perithecia under fluorescent lamps, even though no asci or ascospores were produced.

MUTATIONS

A great deal of evidence had accumulated in the older literature concerning instability and degeneration of cultures (19). A series of papers by Steinberg and Thom (120–122) verified previous claims for chemical induction of mutation in *Aspergillus* and suggested that some mutations might be due to alterations in cell proteins (chromatin, enzymes, etc.) of the fungus. "Injury mutants" showing loss of physiological capacities and morphological abnormalities were obtained from a single spore strain of *Aspergillus niger* and perpetuated unchanged. Other stable strains of *Aspergillus* gave identical results. Chemical induction of mutations was brought about by addition of sodium nitrite to a complete and neutral medium containing ammonium-nitrogen, so that acidity steadily increased during growth of the fungus. Heterokaryosis (40) was not a factor in these results, since a single spore presumably containing only a single haploid nucleus had been used to start the *A. niger* strain. These strains had maintained themselves unchanged for more than a quarter of a century under routine conditions. The mutants of *A. niger,* however, could not be tested through the sexual stage.

More recently, however, the genetic investigations by Beadle and his associates (4) would appear to substantiate the prevailing opinion that actual mutations were taking place. *Neurospora* spores mutate under X-rays, and if the least modified of these are selected and crossed, the states of physiological injury (thiaminless, arginineless, etc.) are transmitted through the sexual stage on a one-gene-one injury basis. Alterations in the enzymes of the cell also appear to play a part in the changed nutritive capacities of *Neurospora* mutants.

The results of investigations by Fries (30, 31) with mutants of *Ophiostoma multiannulatum* have a similar import. Hyphal cells of this fungus are uninucleate, unlike *Neurospora,* and no evidence of heterokaryosis could be found (34). Normal cultures produced 0.03% mutants, and starvation cultures, 0.25 to 0.41% (32). Results of other estimates (33) were X-rays, 1.8% mutation; nitrogen-mustard, 1.3%; normal $< 0.06\%$; caffeine, 1%; and theophylline, 0.6%. *Neurospora crassa* (49) also mutated with nitrogen mustard-control, 0.13%; nitrogen mustard, 3.8%.

Numerous instances of reversion of mutants in cultures have been reported. More recently these include *Aspergillus niger* (121, 122), *Ophiostoma multiannulatum* (31), *Neurospora crassa* (37), yeast (65, 66) and *Neurospora sitophila* (123). The chemical basis for reversion of cultures is still undetermined. Steinberg and Thom (122), as already mentioned, suggested that it consists of repair of chemical injury to cell protein, whereas others are inclined to believe the gene is produced anew. Other explanations may also exist.

A decision between repair and reconstruction of genes in reversions should take into consideration the fact that the gene may be present intact but modified to require altered conditions of operation. A "perithecia-less" *Aspergillus amstelodami* produced by nitrite fruited profusely with double the normal sucrose content (unpublished data of Steinberg and Thom). A pyridoxineless strain of *Neurospora sitophila* grew normally on an acid medium with ammonium-nitrogen (123). *Mitrula paludosa* required biotin with ammonium salt but grew slowly without biotin on nitrate (28). Sartory, Sartory and Wurtz (103) claim production of perithecia, asci and ascospores in *Aspergillus niger* under the influence of radium radiation.

The practical problem awaiting solution is the production and maintenance of stable strains of fungi for nutrition and pathogenicity studies. Studies by the writer on the mineral requirements of fungal pathogens of tobacco (118) had been suspended because of unavoidable degeneration of stock cultures on potato-dextrose agar. They became almost unrecognizable in appearance and almost incapable of growth or pathogenicity. Selection (mass) and propagation on an *Aspergillus niger* optimum solution fortified with six vitamins led to "rejuvenation" as concerned vigor and appearance. *Phytophthora parasitica* var. *nicotianae, Bacterium solanacearum, B. tabacum* and *B. angulatum* could also be maintained thereon. Its formula per liter was: sucrose, 50 gm.; NH_4NO_3, 1.90; KH_2PO_4, 0.55; $MgSO_4.7H_2O$, 0.35; agar, 12.5; biotin, 0.02 p.p.m.; thiamin, 1.0; niacin, 1.0; pyridoxine, 0.5; calcium pantothenate, 0.5; p-aminobenzoic acid, 0.10. Calcium carbonate was usually added to the tubes before autoclaving except those for *Sclerotium.* A small quantity of glutamic acid increased vigor of the bacterial cultures, *T. basicola* and *P. parasitica.* Trace elements were added to this medium but were probably unnecessary because of impurities in the agar. All cultures except those of *Phytophthora* remained viable after four months at 30° C. with parafilm seals.

PATHOGENICITY

A brief but excellent review of this subject has been published by Brown, Brooks and Bawden (11), and in a book by Gäumann (36). The citations that follow deal primarily with a few special products of exosmosis of fungi. No references are included to numerous studies of other products such as enzymes, peptides and acids of fermentation.

Sclerotium rolfsii (48) forms oxalic acid in sufficient quantity to kill the cells of its host. *Helminthosporium victoriae* produced a toxin in culture and on its host, and *Puccinia coronata* one on its host only (67). That of *H. victoriae* produced the same symptoms on its host as does the fungus. It was therefore concluded that susceptibility was induced by toxins. Toxin formation has also been reported for *Ceratostomella ulmi* (137) and *Phytophthora cactorum* (50, 51). Symptoms of Dutch elm disease—*Graphium ulmi*—could be produced by toxins formed in culture (23). Lycomarasmin from *Fusarium persici* caused wilt in the tomato plant. On hydrolysis it yielded glycine, dl-aspartic acid, pyruvic acid and ammonia but no serine (85).

Spores of *Piricularia oryzae* grown on a synthetic medium were found to be as virulent as those grown on the rice plant (56). Leben and Keitt (57) studied the nutrition of four pathogenic and three non-pathogenic strains of *Venturia inaequalis*, the apple-scab fungus. They reached the conclusion that no differences in carbon, nitrogen or vitamin nutrition of the various strains were clearly associated with their pathogenic capabilities.

Availability of stable strains of pathogenic fungi producing toxins should permit of studies relating pathogenicity to nutrition in some of its phases. It is well known that enzymes, amino acids, peptides and organic acids are among the products of exosmosis of fungi. The marked toxicities of amino acids to tobacco seedlings (116, 119), and even of diffusates from non-pathogenic soil bacteria (117), may prove to be an important factor in pathogenicity.

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THE PHYSIOLOGY OF SPORE GERMINATION IN FUNGI

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INTRODUCTION

The physiology of spore germination has interested mycologists since about the middle of the last century, and reports by the pioneer investigators usually included some observations on this subject (5, 7, 28, 41, 65, 67, 69, 70, 153, 154, 155, 158, 171). In 1860 Hoffman (70) published a paper devoted entirely to this field, and de Bary's great book on the morphology and development of fungi (28) contains much information that is still useful to physiologists. In fact, most of the phenomena that concern students today were investigated before the close of the 19th century. Certain new aspects have, of course, since then been investigated, such as enzyme systems and respiratory mechanisms; in addition more critical techniques have been applied in recent researches. Only two important reviews of the subject have been published, one by Duggar in 1901 (37) and the other by Doran in 1922 (32). Since most of the data on spore germination are included in papers devoted primarily to either plant pathology or mildew prevention, the information has been difficult to collate; and since comparatively few fungi have been studied, very few generalizations on spore germination are possible.

Germination is the initial stage in the development of a fungus mycelium from its spore. It differs from the growth of hyphae primarily in the ability of the spore to utilize its stored reserves for metabolism and consequently in the capacity of many spores to germinate either in water or very simple media. Another difference is that germination need not be accompanied by synthesis of protein. Physiologically it is characterized by transformation of a spore from a stage of low to one of high metabolic activity (46, 52, 53, 54, 91, 92, 95), and morphologically by a swelling of the spore and production of a germ tube (28, 41, 51). The term "germination" commonly also includes the release of zoospores from sporangia, and even in this case the spore obviously has changed from a resting to an active metabolic state. As far as has been observed, the process of germination is similar in all types of spores, whether sexual or asexual, and is in general influenced by the same physiological agents which define the activities of other plants or animals.

Various criteria are used to determine the effect of different physiological agents on germination, of which the most frequently used is the percentage of spores that produce germ tubes. Other standards of measurement are the time required for germination, length of the tube and its rate of elongation (96, 144, 145, 161). Tompkins (145) believes that these criteria measure different processes and that all should be evaluated in such studies. Nevertheless even such criteria often can not adequately describe spore responses; certain Urediniae, for example, can elongate very rapidly in water but never form sporidia, while in moist air only short germ tubes are formed but bear sporidia (6). None of the above mentioned criteria would indicate the optimum moisture relations for normal germination which should lead to sporidia formation, and therefore the latter would be the important measure of optimum conditions. When studying any one physiological agent, all other conditions should be as near their optimum as possible, else one of them might be the limiting factor (34). Better still, though more difficult, is the procedure of varying a few factors simultaneously.

WATER RELATIONS

Water is the prime factor in any germinating medium and for many spores is the only substance necessary to start germination (10, 28, 44). Its imbibition causes the first visible symptom of germination, the swelling of the spore, often to more than twice its original size (9, 63, 67, 91, 92, 103, 168), and on further germination the volume of protoplasm can sometimes increase more than ten times (144). Thus absorption of water by spores resembles the imbibition activity of lyophilic colloids (57). This phenomenon, however, is dependent upon some vital mechanism of the spore, for dead spores do not swell and absorption varies with the viability of the spore. The swelling of spores of *Penicillium expansum* changes inversely with the concentration of copper-8-

quinolinol, and high concentrations inhibit swelling entirely. When water has entered the cell, respiratory reactions increase very rapidly (46, 91, 92, 95), even before any germ tube is visible. Similarly copper is not absorbed by *Sclerotinia fructicola* until germination begins (56). Germination often occurs in water without any evident increase in protoplasm, as in *Puccinia graminis tritici* which produces germ tubes 50 times the length of the spore and contains only the protoplasm which has migrated from the spore to the tip of the germ tube (28, 58). *Myrothecium verrucaria,* on the other hand, in the presence of nutrients increases its dry weight during germination, indicating that a synthetic activity can also accompany germination. The properties of water are so unique that not even heavy water can be substituted. Incipient germination of *Erysiphe graminis* occurs in deuterium oxide, but further elongation is inhibited and the final length of the germ tube varies inversely with the concentration. Death does not occur, even after an immersion of 24 hours, and germination continues when the spores are placed in ordinary water (112).

Fungi vary greatly in their water requirements. Some, *e.g., Colletotrichum trifolii, Sclerotinia fructicola* and *Puccinia coronata,* require liquid water (22, 35, 39, 44, 85, 100, 101, 159, 168, 169). For others water vapor is sufficient, as was early recognized by many early French and German mycologists (7, 28, 70, 79, 137, 147, 148), though usually this group of organisms will also germinate in liquid water. Tompkins has postulated that only the vapor phase of water is active, since some spores germinated equally well or better in a saturated atmosphere than when floating in water and hardly germinated if submerged (28, 65, 79, 144, 145, 148). Rippel (116) believes that the decisive factor is the gradient between the humidity of the air and the spore membrane. Usually the greater the humidity the greater the number of spores which germinate. Some fungi, *e.g., Peronospora nicotianae,* require 100 per cent relative humidity (55, 165). *Puccinia triticina* and *Venturia inaequalis* will not germinate below 99 per cent humidity, while *Alternaria brassicae* and *Penicillium chrysogenum* need at least 91 and 81 per cent, respectively (9, 20, 22, 49, 50, 59, 62, 79, 85, 116, 137). Powdery mildews are unique in their low moisture requirements. Some, *e.g., Erysiphe polygoni* and *E. graminis tritici,* can germinate at 0 to 100 per cent humidity, while others need humidities of about 90 per cent (11, 28, 85, 168). The imbibition capacity of the spores must be very high; Brodie and Neufeld (11) reported osmotic pressures of 60 to 90 atmospheres in the oidia. At very low humidities, however, germination is not normal and the spores soon die; usually at least 75 per cent relative humidity is necessary for good germination and some species even require liquid water (169). Numerous investigations have shown that as a group the powdery mildews germinate poorly in water (25, 35, 85, 166).

Data on the germination of spores near the saturation point are difficult to evaluate unless the temperature has been very closely controlled, for at high humidities a slight drop in temperature will cause precipitation of water. Critical experiments by Clayton (22) and Longree (85) indicate that even at high humidities some fungi can not germinate but require free water, while others are perfectly adapted to utilize water vapor. The optimum need not be 100 per cent relative humidity, and in some fungi it is dependent upon the temperature (9, 62). For *Aspergillus niger* the optimum relative humidity at 30° C. is 100 per cent, while at 40° C. it is 93 per cent. Humidity also effects the temperature range at which germination takes place; the higher the humidity the wider the range (144). The enhancing effect of increased relative humidity on germination is indicated not only by the greater number of germinating spores but also by the time of germination, rapidity of elongation and final germ tube length.

NUTRITION

That water alone is insufficient for the germination of many fungi was recognized very early in the development of mycology (28), since improved germination could usually be obtained by addition of natural products to the medium (12, 14, 28, 153). *Urocystis tritici,* for example, does not germinate in water or ordinary nutrient solutions, but will if presoaked in the presence of wheat seeds (106). Similar stimulatory effects have been reported for peptone on *Phycomyces rutens* (142) and for yeast on *Myrothecium verrucaria* (91). Brown (13) has shown that spores of plant pathogenic fungi are stimulated by the exosmosis of material from the leaves and that the effect is greater on attenuated and old spores; even the mere presence of bruised leaves at a distance from the spores can increase germination. This phenomenon has been observed with many other materials, such as wine, apple juice, tomato juice, potato, extracts and mycelia and soil (12, 14, 41, 82, 89, 91, 115, 117, 118, 119, 120, 121, 124, 144, 153). Because often none of the known nutrients could be substituted for such materials, the effects were usually attributed to growth-promoting materials. Nevertheless, none of these effects has been demonstrated to be due to the presence of the known vitamins (90, 91). Other growth promoters, such as hypoxanthine from potato, and guanine, have been shown to be effective for *Phycomyces* (120, 122–124).

Many fungi germinate better in the presence of ordinary nutrients and are sometimes even entirely dependent on an external source of such materials. DeBary (28) points out that in *Mucor stolonifer* and other species, the presence of external nutrients allows the germ tube to grow like a vegetative hypha, for the spore retains its protoplasm and new protoplasm is synthesized as germination proceeds. Quantitative measurements on *M. verrucaria* support these observations, for the dry weight of germinated spores increased after germination (91, 92). Of the materials of known constitution, carbohydrates, such as glucose and sucrose, have stimulated germination most frequently (60, 80, 81, 89, 99, 151). Nitrogen sources have also been implicated for *Penicillium digitatum, P. glaucum, Phycomyces rutens* and *Glomerella cingulata* (81, 94, 142, 143). The requirements for germination are sometimes very specific, as in *P. digitatum,* for which peptone and ammonium but not nitrate ions are effective. Mutants of *Neurospora* which have lost the capacity of synthesizing some amino acids also require the presence of these acids for normal germination, and in their absence germ tubes are produced only after long periods of time (127, 128). Very few studies on the salt requirements for germination have been published, and variable results have been reported for potassium and magnesium (6, 37, 65, 81).

While some spores, *e.g.,* those of *Fusarium lini, Puccinia graminis tritici* and *Stemophyllium sarcinaeforme,* apparently require no external source of nutrients, this independence has not been definitely established for most species (10, 37). Spores can vary quantitatively in their nutritive needs, and only those organisms which are greatly deficient in one or more nutrients are usually noticed; others which are less deficient may obtain their requirements as contaminants from the media on which they are produced. Washing such spores can prevent germination by removing important nutrient materials (56, 81). The critical studies of Lin (80, 81) deserve special mention in explaining the necessity for nutrients in germination. Spores of *Sclerotinia fructicola*, which were capable of 85.5 per cent germination when collected by brushing a culture or flooding it with water, failed to produce germ tubes when thoroughly washed and centrifuged. Since similar results were obtained when the spores were collected by gentle suction, the effect of the washing obviously was not due to leaching essential materials from the spore but merely to the removal of the contaminants. Addition of dextrose, sucrose, galactose or other carbohydrates to distilled water resulted in almost perfect germination, indicating that *S. fructicola* does not store sufficient carbohydrates in its spores. These spores are also capable of germinating, though to a lesser degree, under anaerobic conditions, which suggests the presence of a fermentation mechanism during germination. Similar experiments with *Glomerella cingulata* revealed that its spores lacked not only an energy source but also nitrogen, phosphorus, magnesium and sulfur. Sufficient potassium must have been stored in the cell, since no external source was required. *Coccomyces hiemalis* might also be deficient in a number of nutrients, for Magie (89) reports that, though germination is improved in the presence of sugar, addition of salts further increased it. Various scattered reports indicate that for some species of fungi germination is increased as the concentration of spores is increased (8).

OXYGEN AND CARBON DIOXIDE

While oxygen may be indispensable for the germination of fungus spores, comparatively few exacting experiments to support this concept have been reported and most of the evidence consists of indirect observations (74, 155, 167). Many observers have agreed that spores commonly do not germinate or produce abnormal germ tubes when submerged in water, but germinate readily when floated on it, as *Melampsora lini, Puccinia coronata* and *Urocysti tritici* (7, 28, 35, 64, 105). Yet such observations do not prove that oxygen is essential; other factors might be responsible for these results, such as improper interfacial tension, the necessity of having water in a gaseous phase (144) or the lack of a gradient between the moisture content of the spore and its environment (116). Nor can the use of carbon dioxide to displace air conclusively indicate that air is vital for germination (114), for such effects could readily be ascribed to the toxic action of carbon dioxide (13, 89, 111). Similarly the inability of *Septoria apii* to germinate in sealed capillary tubes can not be accepted as proof of its oxygen requirement (23). More direct evidence of their oxygen requirement is available, however, for a number of fungi from experiments in which oxygen was removed by aspiration, adsorption by pyrogallol or replacement with nitrogen. *Neurospora tetrasperma, Ustilago avenae, Coccomyces hiemalis* and *Actinomyces scabies* all failed to germinate in the absence of oxygen, and when the spores were again exposed to air, normal germination occurred (79, 89, 53). Uppal (151, 152) demonstrated the importance of oxygen for germ tube formation among the Peronosporales. Such species as *Phytophthora infestans* and *P. palmivora,* which germinate directly at high temperatures but indirectly at low temperatures, and those species which invariably germinate directly, required this gas, but when *P. infestans* and *P. palmivora* were germinated at low temperature with the formation of zoospores, no oxygen was needed. Other fungi as *Plasmopora viticola,* which produced zoospores only, also required oxygen to germinate. According to Lin (80), *Sclerotinia fructicola* also can germinate under anaerobic conditions; the percentage of germination is reduced under these conditions, however.

Associated with germination is a sharp increase in oxygen consumption, for even resting spores utilize small but measurable amounts of the gas. The uptake of oxygen by *Sclerotinia fructicola* is increased as the spores are wet, and is further increased in a sugar solution (46). With this fungus respiration was further augmented at the time the germ tube appeared, but no such secondary increase occurred with two other fungi, *N. tetrasperma* and *M. verrucaria* (46, 52, 53, 54, 91, 92). Differences in oxygen tension are reflected in the Qo_2, and within certain limits increased oxygen concentration is accompanied by an increased rate of uptake (52, 91). According to Brown (13), germination of *Botrytis,* *Fusarium* and *Alternaria* spores is not affected until the oxygen concentration is reduced to less than one per cent. All of the spores had a low rate of endogenous respiration but rather high exogenous respiration rates. Spores of *M. verrucaria* can oxidize a great variety of substrates in this process, including carbohydrates, amino acids, organic acids and alcohols (92). Sucrose and mannose are most readily utilized by *M. verrucaria,* and in their presence the Qo_2 is about twice that of glucose, while with arginine, histidine, aspartic acid, ethylene glycol and urea no oxygen was consumed (92). Synergistic effects of mixtures of various carbon compounds were never observed with this fungus. The respiration of *N. tetrasperma* is different from that of the other two fungi, since the young ascospores are strongly dormant and thus even in water have a low oxygen consumption, but upon heat activation of the spores the incidence of germination is reflected in a greater than ten-fold increased respiratory quotient (52, 53).

Carbon dioxide has a deleterious effect on germination at high concentrations, but at low concentrations may be beneficial. Its inhibiting concentrations are dependent to some degree upon the fungus and the medium, as in *Botrytis cinerea* which is inhibited at 20–30 per cent carbon dioxide in water but not until 50 per cent in a nutrient medium (13). Morphologic changes also occur in its presence; *Rhizopus nigricans* at 50 per cent carbon dioxide germinates with stunted enlarged cells which will, however, resume their normal appearance when removed to air (53). *Coccomyces hiemalis* is so sensitive that even the amount of gas emitted from crushed leaves in a closed chamber prevents germination (89). Usually, for short periods, carbon dioxide is not lethal and teliospores of some rusts can be exposed for 24 hours and still germinate, but different spore forms of the same species can vary in the sensitivity, such as the teliospores and the sporidia of *Gymnosporangium juniperi-virginianae* (114). The toxic effects of carbon dioxide might be direct, as in replacement of oxygen, but Platz and Durrel (111) have shown that increased carbon dioxide concentration may have indirect effects by raising the hydrogen ion concentration. A concentration of 30 per cent carbon dioxide increased the hydrogen ion concentration of water about 20-fold. Both stimulating and toxic effects can occur with the same organism, as with *Basisporium gallorum* and *Ustilago zeae;* germination increased as the concentration of carbon dioxide rose from 5 to 15 per cent carbon dioxide, then decreased until it was almost entirely absent at 50 per cent.

TEMPERATURE

The effects of temperature upon germination have received more attention than any other environmental factor, due to their indirect influence upon the infection of economic plants by fungi. Not only is the percentage of germination affected, but also the time for germination and the rate of elongation of the germ tubes. The modifying nature of heat is reflected in almost all physiological processes primarily by changing the rate of cellular reactions; increased temperatures cause more rapid biochemical reactions and decreased temperatures slow them down. *Neurospora tetrasperma,* for example, consumes more than twice the amount of oxygen at 25° C. as at 15° C. (54). Physiologically similar changes are seen in the time required for germination (160) and in the rate of elongation. For *Lenzites sepiaria* this elongation of the germ tube increased from 0 microns at 16° C. to 400 microns at 32° C. during a 20-hour period (136). Most of the reactions of spores are correlated with a gradual increase in temperature, though sometimes a critical temperature must be reached before any effects can be measured. Thus in the activation of some dormant spores a temperature of about 50° C. must be attained for a short time to start some reactions which allow germination and respiration to proceed (33, 52, 133).

While no complete explanation of thermal effects on fungi is possible, certain general information of heat effects on biochemical systems is useful in understanding the physiological responses of the spore. Enzymes are materials which control most of the known living processes (57) and are very exacting in their temperature requirements with different optimum temperatures for the various enzymes. Pectinase production has a different optimum in various fungi, and these optima are different in turn from those conducive to the general growth of the organism (51, 161). Similarly the enzyme systems responsible for adenine synthesis in a mutant of *Neurospora* are active at 25° C. but not at 35° C. (97). Deleterious effects of temperature are caused not only by inactivation of enzymes but also by an unbalanced production, causing accumulation of some toxic materials. Since imbibition of water is usually essential for germination, its intake could be limited by the effect of heat on the viscosity of water; between 0° C. and 35° C. the specific viscosity decreases 60 per cent. Finally very high temperatures can coagulate the proteins of the spore and cause death. Exposing spores to temperatures above their maximum for germination for a prolonged period causes complete loss of viability (55, 59, 83, 84, 114, 165). But the optimum temperatures and the thermal tolerance are not absolute but depend on other factors, such as humidity, pH and even the temperature at which the spores were produced (9, 62, 83, 124, 144).

The results of exposure to low temperature are rarely lethal by their direct action on chemical systems, for they merely retard reactions, and under proper conditions spores can remain viable for years. From analogies with bacteria the lethal effects of extreme cold could be attributed to the formation of ice crystals in the cell or on the membrane as the temperature rises toward the freezing point of water (162). Lethal temperatures vary with the different species, and most chlamydospores and such heavy walled spores as teliospores and zygospores can withstand very low temperatures (29, 110). Teliospores of *Puccinia graminis tritici, P. graminis avenae* and *P. coronata* and the perithecia of powdery mildews of grain (19) overwinter in Minnesota and the neighboring States where temperatures of −30° C. are not rare. Even conidia of *Glomerularia lonicera* can withstand temperatures of −7° C. for more than 40 days (59), and *Peronospora destructor* can be frozen for more than 15 hours and yet remain viable. In higher plants this capacity to survive low temperatures has been correlated with an accumulation of sugars in cells, changes in protein colloids, decrease in free water and variations in the permeability of the cell membranes.

More data are available on the cardinal temperatures for germination of the fungi than any other environmental factor, and a survey of the data on more than 80 species showed great variation for different species, strains and media. Generally the minimum temperature is between 0° and 10° C., though an occasional organism, *e.g., Sporotrichum carnis*, will germinate at less than 0° C. (63). Only two organisms required temperatures greater than 10° C. At low temperatures the period of observation is very important, for germination often occurs very slowly (63, 160). The optimum temperature, however, is independent of observation time, and the peak of the curve remains the same. Among this representative group of fungi the optimum temperature varied between 15° and 44° C. The distribution follows the pattern of a normal curve with a peak of 30 species in the class 24° to 28° C. The classes 21°–25° C. and 26°–29° C. contain 25 and 27 species, respectively, giving a rather broad topped curve. On each side of these two classes a five-degree change reduced the fungi in half to 12 and 13 species. *Puccinia antirrhini* (90) had an optimum at about 10° C., and four fungi between 36° and 40° C. The maximum temperature also varies greatly, and some of the thermophilic organisms, such as *Rhizopus chinensis,* germinate as high as 52° C. (160).

Different species have different ranges of temperature in which they will germinate. Some are very narrow, as for *Puccinia antirrhini* which produces germ tubes only between 5° and 25° C. and *Cronartium ribicola* between 8° and 25° C. (35); in contrast, *R. nigricans* has a range more than twice these, between 1.5° and 45.5° C. (160). Generally a deviation from optimum conditions narrows the limits of germination (35). Temperatures at which the spores are produced, the pH, the humidity and the constitution of the media can alter the cardinal temperatures slightly. No correspondence between the four classes of fungi and differences in temperature requirements could be formulated for the various species studied, but the rusts usually did best at about 20° C. A comparison of the cardinal temperatures for germination and growth of 28 fungi revealed a close conformity, and often the temperatures for these processes were identical (1, 82, 84, 85, 136, 138, 149, 157, 160), though occasionally wide differences, as with macroconidia of *Chalaropsis thielavioides,* have been reported (86).

HYDROGEN ION CONCENTRATION

Another limiting factor for germination is the hydrogen ion concentration of the environment, though under natural conditions this is not important because extremely acid or alkaline conditions rarely occur. As a group, the fungi tend to be acidophilic with an optimum pH in the acid range, and only rarely is germination favored by an alkaline reaction. The optimum for many species is about a pH 3.0 (94, 156) and for others between pH of 6.0 and 7.0 (45, 89, 94, 140, 156). *Colletotrichum gossypii* is one of the unusual fungi because of its basophilic character, but the germination ranges of many other fungi also extend far into the alkaline regions (156). Differences between species are sometimes very distinct, as in the case of *Penicillium italicum* which produces germ tubes between pH 2.0 and greater than pH 10.0 (94), and *Venturia inaequalis* which germinates only in the range pH 2.6 to 6.8 (107). Although the acidophilic character is predominant among those fungi which have been examined, Westerdijk (163) disagrees with this general belief, for, from her wide experience with the culture collection at Baarn, Holland, she has found that neutral media are best for the growth of fungi. In some species, *e.g., Sclerotinia fructicola* and *Fomes annosus,* double maxima have been reported (140); usually the greatest amount of germination occurs in the acid region, while a second peak occurs near the neutral point. Tilford (140) attributes double maxima to the isoelectric point of the protoplasmic proteins, for at this pH metabolism is at a low point, whereas on either side physiologic activity again rises. Differences in temperature or time of observation do not affect the optimum pH, however, and the nature of the medium sometimes has a marked influence on the maximum pH value. Not only is germination influenced by hydrogen ion concentration but it also can change the pH of the medium, and usually this change is toward neutrality (156).

ENZYMES

Since most metabolic processes have been associated with enzyme activities, the presence of such catalysts in germinating spores can reasonably be assumed. A number of such systems have been demonstrated in studies on the respiration of spores of *Neurospora.* Carboxylase is present in the activated spores, a phaeohemin oxidase also seems probable, and the effects of some respiratory poisons indicate other respiratory catalysts. In addition the flavin enzymes may also be responsible for a small part of the spore's respiration (52, 53, 54). 55 56 57 Two other enzymes related to oxygen physiology, catalase and peroxidase, have been found in spores of *Aspergillus oryzeae* (136). Studies on that

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fungus (136) have demonstrated the activity of a number of other enzymes, urease, glucosidase esterase, nuclease, invertase, amylase, chymase, phytase and peptase, but seven other enzymes could not be found in spore extracts. Pectinase and amylase are present in at least two species of *Rhizopus* and sucrase is present in some Aspergilli (76, 77, 136), in *Penicillium glaucum* (92) and in *Myrothecium verrucaria* (91, 92). The last fungus shows some strange anomalies, for, although hydrolysis of neither maltose nor cellulose could be experimentally measured, this fungus will grow on both substrates. Mandels (92) infers from this that the spore must be in actual contact with cellulose in order to germinate.

Extensive researches have been made only with the sucrase of *M. verrucaria,* for which quantitative measurements of hydrolytic activity were used. Mandels (92) showed that sucrase is not secreted by the spore into its medium, nor can it be leached from living or dead cells; it is released when the spore is ruptured. Yet the enzyme is not adsorbed on the spore wall, for no elution procedures were successful, and he postulates its position near the surface of the spore, perhaps in the cytoplasmic membrane. Sucrase activity is constant for four to five hours in a phosphate buffer, but if yeast extract which promotes spore germination is added, an increased activity takes place after only one hour. Synthesis of this enzyme is apparently dependent on the presence of sucrose and is initiated in the swelling stage of germination. Its release is correlated with an increase in spore volume and follows the beginning of protoplasmic synthesis. Hydrogen ion concentration affects the hydrolysis of sucrose by sucrase similarly in vitro and in vivo.

DORMANCY

Not all viable spores will germinate under optimal environmental conditions and many remain dormant for long periods (7, 28, 37, 93, 110, 114, 124, 171). Recently a broad concept of dormancy has been proposed which includes the inability of a living spore to germinate because of any environmental or nutritional factor, such as lack of nutrients, moisture or proper temperature (91). In this discussion, however, a more limited definition will be used in which dormancy applies only to the innate state of the cell; a spore is dormant when it does not germinate under the same nutritive and environmental influences which later allow production of germ tubes. In some cases dormancy is definitely related to the maturity of the spore (130), as withurediospores which do not germinate if scraped from the sorus before they are loose but must first age, while mature spores germinate immediately (100). Similarly ascospores of *Pseudopeziza trifolii* and *P. medicaginis* germinate readily when mature and naturally discharged but not if they are obtained by crushing the ascus (73). Some spores are killed if detached before maturity, but others can continue their development apart from the parent mycelium (35), while spores of *Sordaria fimiseda* can germinate even before completion of their definitive membrane (28).

Different types of spores from the same species usually have different dormancies; thus the urediospores of *P. graminis tritici* germinate readily, while the teliospores require about six months aging (26, 58). Conidia generally germinate with only a short rest period or none at all, but the ascospores of the same species remain inactive for long periods (127, 128, 133). Considered as a maturity phenomenon, dormancy is a common property of all spores, and only the length of time for maturity varies with the species and spore types. Many spores need long rest periods, and in certain species of the Ascobolaceae germination is rarely observed. As has been mentioned, the teliospores of many rusts are dormant for long periods. Even more refractory are the chlamydospores of the smut *Ustilago striaeformis* which requires a resting period of 285 days (27). The zygospores of aquatic fungi generally require long rest periods, as in the Saprolegniaceae where the time for maturation varies from 30 hours to at least three months, depending on the species (37, 78, 131, 133, 170). One feature which some spores with long dormancy periods have in common is the presence of nuclear changes, such as fusion in the zygospores, fusion and division in the teliospores, and in the Ascobolaceae formation of ascospores by complex nuclear divisions.

Three types of treatment have been used to shorten the rest period of spores. The first of these attempts to simulate natural environmental conditions, such as alteration of temperature and humidity, a procedure that has been moderately successful for the rusts and smuts (26, 52, 106, 113). The second method is a chemical treatment, usually with dilute solutions of organic compounds, of which the aldehydes, benzaldehyde, salicylaldehyde and furfuraldehyde have been most successful (42, 106). Organic acids, esters and mineral acids are also sometimes effective (12, 42, 82, 106, 125, 151); the effect of the acids is not correlated with their hydrogen ion concentration. It is interesting that ten years elapsed between the time when Goddard (53, 54) stated that no chemical method of stimulating the germination of *N. crassa* was known, and the report of Emerson (42) that furfural will activate these spores. The third method is heat shock which has been effective both for a number of species of the Ascobolaceae and for *Phycomyces* (33, 133). More than one type of treatment can sometimes break dormancy in the same spore; heat activation or furfural is effective for *N. crassa,* while alternation of dry, wet, hot, and cold conditions, or benzaldehyde will induce germination in teliospores of *P. graminis tritici* (42, 106).

Though different mechanisms have been suggested to explain the artificial breaking of dormancy, such as activation of enzyme systems or change in permeability of the cell membranes, the only experimental results available are from studies on *Neurospora crassa*. These ascospores are activated by short exposures at 49°–52° C.; after this treatment germination increases from about one per cent to 95 per cent. Heat treated spores can be deactivated by placing them under anaerobic conditions or in cyanide, but reheating them will again stimulate germination (53). In contrast, furfural activation is irreversible (42). Activation itself is not dependent on the presence of oxygen, but consumption of this gas is greatly increased after heat treatment. Another difference between activation and germination lies in their response to cyanide; the activation mechanism is insensitive to this reagent, while respiration of the germinating spore is blocked by cyanide. No carboxylase activity is present in the dormant spore, whereas this enzyme is functional in activated cells. From his studies, Goddard (54) suggested that "two qualitatively different respiratory systems are present, namely, the dormant system which functions in the absence of carboxylase, and a second system, active in heat treated spores, which passes over the enzyme carboxylase. The respiratory block is then the inactivity of the enzyme carboxylase".

RADIATION

Radiation often alters the behavior of spores, sometimes by inhibiting germination, and more rarely by stimulating the process. These results are related directly to the time of exposure and to the intensity of the rays; and usually the inhibitory activity increases as the wavelength decreases through the visible and ultraviolet regions (38, 71, 108).

If any generalization can be made of the result of exposure to visible light, it is that most spores are not materially altered by diffuse or low intensity light (17, 31, 32, 34, 64, 68, 70, 72, 98, 114, 115, 135, 154). The effects of high intensity artificial light or sunlight have been more difficult to appraise because, first, the direct radiant effects have not always been separated from the effects of light in raising temperature (36), and second, in the case of sunlight no mention has been made whether or not the spores were covered with glass. If they had been covered, the consequence of the ultraviolet component of sunlight could not have been appraised. Many reports indicate that strong artificial light is deleterious to some spores (24, 68, 72, 87, 135, 153). The result of such irradiation, however, is sometimes dependent on the percentage of moisture in the spore and on the temperature or pH of the surrounding medium (5, 24, 30, 31, 108, 109, 134). Cochran (24) has shown that exposure of *Phragmidium mucronatum* spores on dry slides to artificial light does not hinder subsequent formation of germ tubes, whereas the process was depressed when the spores were exposed on water agar. Similar effects have been shown with ultraviolet light exposure on *Puccinia graminis tritici* (32). Direct exposure to sunlight is debilitating to many spores (31, 114, 135, 165) and occurs even when the heating effect is prevented; for example, exposure of sporangia of *Peronospora nicotianae* for one hour on a block of ice is lethal to that organism (165). The effect of time of exposure is illustrated in studies on some wood-decaying fungi in which spores are inhibited by a one-day exposure but are killed by a two-day treatment (135). Not all fungi are injured by sunlight; such divergent species as *Cronartium ribicola*, *Ustilago destruens*, *Trichoecium roseum* and *Penicillium glaucum* can withstand irradiation which injures many other species (36, 70, 108). Not all wavelengths of the visible spectrum are equally fungicidal, and blue light appears as the most active portion of the visible spectrum (38). Sunlight can inhibit germination of urediospores even when passed through orange, red, yellow or purple filters (31).

Some of the early investigators reported stimulatory effects of light on germination; most of these reports have not been confirmed. Hoffman (70) reported such effects with *Agaricus campestris,* but Ferguson (44) noted not only the absence of stimulation but the presence of a depression of germination. Better germination after irradiation has been found for some of the oomycetes, yet again the opposite result was reported in the studies of Melhus (98). Nevertheless the enhancing properties of radiation must not be dismissed, for recently Ziegler (170) stated that light stimulated zygospore germination of *Protoachlya hypogena, Achlya recurva* and *Thraustotheca primoachlya,* and similar stimulation has been shown to occur with exposure of *Colletotrichum phomoides* to certain regions of ultraviolet range (71).

Positive phototropism of germ tubes has never been conclusively demonstrated for any of the species studied (19, 24, 135), but a negative response has been reported for a number of rusts (45, 47). Forbes (45) showed that negative tropism to white light is due to its blue and violet components.

As would be expected, spores are more readily harmed by ultraviolet and X-rays than by visible light, and the injury varies in the same direction as the intensity of the rays, though the decrease in germination is not always proportional to the increased intensity (30, 36, 43, 88, 108, 126, 134). According to Rodenhiser and Maxwell (126), chlamydospores of *Ustilago hordei* were not injured by exposure to 100 Kr; an increasing number of spores failed to germinate between 100 and 1000 Kr; and above 1000 Kr all the spores were killed. Sporidia production ceased above 150 Kr, and increased germ tube length was noticed between 60 and 150 Kr. X-rays are also very lethal to spores, and, for the ascospores of *Neurospora* at least, Uber and Goddard (150) state that death does not result from a single well defined reaction but from several deleterious processes.

One protective mechanism against injurious rays is offered by the pigments of some spores, and many correlations between color of spores and resistance to radiation have been made (38). Weston (31, 32) showed that white and orange spores of *Puccinia* *graminis tritici* are more easily killed by ultraviolet light than are grey and red spores. Studies on human pathogens by Chavarria and Clark (18) and by Nagy (104) on ten different fungi also indicate that the darker the color the more irradiation is necessary to kill the spore.

TROPISMS

Though the tropic responses of germ tubes were studied by a number of investigators in the late nineteenth and early twentieth centuries, very few features of this phenomenon have been widely accepted. Myoshi (102) observed both positive and negative chemotropisms, such as growth toward sugar solutions and away from acids and alkalis, but neither Fulton (48), Corner (25) nor Clark (21) could confirm this with their fungi. However, Graves (61) found a weak positive chemotropism in *Rhizopus nigricans* toward turnip juice, sucrose and glucose. The one definite and widespread reaction of germ tubes on which most investigators agree is their negative response to the presence of other hyphae, for the germ tubes in their studies turned away from openings or media which contained other germ tubes and even from media containing their own staling products (21, 48, 61). As has been discussed in a previous section, negative phototropism among the rusts is also widely accepted. Thigmotropic reactions of germ tubes have also been demonstrated for the powdery mildews (25).

TIME FOR GERMINATION

The time required for production of germ tubes in any collection varies greatly, even when mature spores are tested and other factors are kept at their optimum. With *Sclerotinia fructicola* as much as ten hours can intervene between the time the first spore and the last one germinate in a microscope field. Another critical feature is the criterion on which time is recorded; for some investigators it is the moment when a swelling in the spore wall appears, for others it is the time at which the germ tube has parallel walls, and a third group uses the time when the germ tube equals the length of the spore. The period is greatly affected by temperature (3, 141), and therefore the time for germination has also been used in determining the optimum temperature for this process (160). The internal conditions, such as maturity of the spore, in addition to the innate propensity of the species are naturally also important in regulating the speed of germination. Doran (35) in 1920 compiled the literature on 25 different fungi, and data on others have been published since then. Of the latter group, for example, *Melampsora lini* germinates rapidly within 45 minutes, *Diplodia zeae* slowly within 13 to 16 hours, while *Pyrenophora nicotianae* is intermediate, requiring two hours (17, 64, 75, 139, 146, 165).

SENESCENCE

Spores of fungi undergo not only a period of maturation during which the percentage of germinating spores increases but also a period of senescence which is accompanied by decreasing vitality. Senescence should begin immediately after the peak of germination has been reached, but data on this phase of germination are very meager. Most studies on longevity are based on the assumption that the spores were collected when mature, an assumption which is unwarranted with such organisms as the telial stage of wheat stem rust in which about a six months rest period is common after they have been collected. The viability of all spores gradually decreases and is dependent both on the inherent characteristics of the organism (125) and upon environmental conditions (2, 35). Unless stored under special conditions some spores are extremely short lived. According to Doran (35), Spaulding reports that sporidia of *Cronartium ribicola* lived less than ten minutes, and Reed and Crabill (114) observed that the sporidia of *Gymnosporangium juniperi-virginianae* are killed in three to five days in air. Conidia of *Peronospora destructor* are viable for only 3 days at 15° C. and 75 per cent humidity. In contrast some sporidia of *Ustilago zeae* have remained viable for at least three and one-half years, chlamydospores for five years (110). Roberg (125) tested desiccated cultures of 14 species of *Aspergillus* after 10–15 years and successfully cultured all but three of them, though several races of the same species differed in longevity. He concluded that longevity is primarily an hereditary trait. Records of the viability of many other spores have been reported and indicate a great difference in survival potential but always a gradual decrease of viability with time (17, 35, 38, 55, 59, 66, 89, 93, 101, 110, 113, 114, 135).

Temperature greatly influences the longevity of spores, and long senescence is correlated with low temperatures, while high temperatures shorten this period (2, 84, 110, 135, 157, 165). The loss of viability is especially rapid above the maximum temperature for germ tube formation (83). According to Gould (59), *Glomerularia lonicera* was viable for only 15 days at 15° to 25° C. but more than 40 days at −7° to 7.5° C., and similar results have been reported for *Exoascus deformans.* When spores of *G. juniperi-virginianae* were dried at different temperatures from 13° to 26° C., the viability decreased inversely with temperature. Low temperatures, often freezing, have no deleterious effect on most fungi (19, 100). Other environmental factors can sometimes change the normal temperature relations of spores, as Ling (83) has shown with species of *Colletotrichum* wherein the thermal tolerance increased slightly with an increase of the incubation temperature of the parent culture and also as the pH of the medium was raised.

Humidity also influences the length of time during which spores retain their vitality, and high humidity tends to shorten this period (2, 3, 135). Such effects were evident in studies of Rosen and Weetman on *P. coronata,* even at low humidities, for at 50 per cent relative humidity all the urediospores were dead after six months to a year, but at 25 per cent they remained viable for more than a year. Nevertheless no such deleterious effects have been reported for a number of species, among them *Actinomyces scabies* which was unaffected by moisture between 12° and 30° C. (129). Duggar (38) kept *Aspergillus flavus* on distilled water for 90 days during which no germination occurred, yet these same spores germinated readily when placed in a nutrient solution. For some of the rusts the optimum humidity as determined by two laboratories is 49 per cent over a wide range of temperature (113), and aeciospores of *Cronartium ribicola* have been reported to survive longer in moist air than in dry air. Desiccation delays the senescence of some spores, as *U. zeae* which survived five months drying (110), but is injurious to other spores (3, 66), though the latter effect might involve a different phenomenon than a continued absence of water. Rapid desiccation at low temperatures, on the other hand, is favorable for the survival of most fungi. A widely fluctuating environment is most injurious to spores, and most mature spores germinate very poorly or not at all when alternately wet and dry or frozen and thawed (19, 110, 135).

Some evidence indicates that spores are naturally protected to some degree from the debilitating effects of environment and that studies on glass slides might not accurately describe the situation. This is evident with pycniospores of *Endothia parasitica* which germinated readily even after the horn had been stored for one year, but if its gelatinous matrix was first dissolved, viability was entirely lost within one month (66). A similar protection by a water-soluble coating on the spores occurs in *Glomerella rufomaculans* (16). When spores are enclosed in a fruiting body, such as a perithecium, they retain their viability longer than when removed from the ascus and stored on glass (4, 17). Organic matter of many kinds, including soil, will also protect spores from the loss of their power to germinate (66), and even plant parts such as leaves have been shown to preserve spores; conidia of *Pyrenophora bromi* germinated after longer periods on dried leaves than on glass slides (17).

Recent studies by Mandels (91) indicate that the dry weight of *M. verrucaria* decreases with age, and, since spores respire even when not germinating (52, 91, 95), some of the reserve materials of the spore evidently are oxidized. Supporting this conclusion is the observation that the germination of old spores is stimulated more by the presence of external nutrients than is that of young spores (14). Thus those conditions which increase the metabolism of the organisms, such as high temperature and humidity, do not favor the longevity of spores; conversely low temperature and humidity, which decrease the rate of metabolism, increase the life span of spores because of the conservation of its stored food reserves.

SUMMARY

Water, either in its liquid or vapor phase, is essential for the normal germination of all fungus spores, but the species differ in this requirement. Generally germ tube formation increases with increasing humidity and some species need liquid water. *Erysiphe* is unique, since some species can germinate at zero humidity, yet even such species require vapor for normal germination. For spores which are deficient in food reserves, an external source of nutrients is necessary, while others which have a full complement of nutrients can germinate readily in distilled water. Old spores in which the reserves have been depleted are also stimulated by addition of nutrients to the media. Oxygen is normally required by spores even when resting, but the uptake increases both when germination begins and also if external nutrients are available. Carbon dioxide, on the other hand, is usually injurious, though stimulatory results have also been observed; some of these effects can be attributed to the increased acidity produced in its presence. Temperatures below 10° C. usually greatly retard germination, and the optimum for most fungi is between 15° and 30° C., while above this range the process is inhibited. Some fungi, however, are thermophilic and will germinate at temperatures above 50° C. The production of germ tubes is usually enhanced in an acid medium but is also good when media are near the neutral point; only occasionally does an organism have its optimum in the alkaline region. Various respiratory and hydrolytic enzymes have been demonstrated in spores, and secretion of sucrase during the germination has been established. The dormant period of spores has been shortened or broken by heat, chemical agents and alteration of temperature and humidity. Diffuse light does not influence germination, but sunlight or intense artificial light can inhibit this process and kill the spores. Ultraviolet and X-rays are lethal at high intensities or for long periods, and even when not lethal they can induce mutations. Pigments in the spore walls act as a protection against such deleterious effects. During germination the spore tubes tend to grow away from each other and are "negatively tropic" to their own staling materials. Negative chemotropism is more common than positive chemotropism, though the germinal hypha of some spores will elongate toward nutrient materials. Maturation of spores normally occurs while the spores are on the parent culture, but some spores can complete the process when removed from the sporophore. Later, senescence takes place, with gradually reduced germination, until eventually viability is entirely lost.

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UPPER CARBONIFEROUS AND LOWER PERMIAN CONIFERS

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The transition from the Carboniferous to the Permian and the Permian period itself constitute together one of the most interesting phases in the history of the plant-world. In this the conifers superseded the Cordaites and began for the first time to play a more conspicuous rôle in the forests of the Northern Hemisphere. The occurrence and wide distribution of coniferous remains in the Upper Carboniferous, and particularly the Lower Permian strata, have been known for more than a century, but until recently they were little studied from the morphological and taxonomical points of view. In 1938–1945, however, the author published a monograph on these conifers based on an investigation of practically all the material collected in various countries—including the United States and Canada—since the beginning of the nineteenth century [Florin: "Die Koniferen des Oberkarbons und des unteren Perms", Parts I–VIII.—Palaeontographica 85 B: 1–729, *1–72*, pl. 1–186, f. 1–65, 2 maps. Stuttgart (Germany) 1938–1945]. This material was very copious but consisted almost exclusively of compressions and impressions buried in sediments of various kinds. The cuticular analysis has proved to be of great value in connection with the discrimination and delimitation of genera and species, and to the study of the morphology of the reproductive organs. The results obtained are of special interest to the long debated problem of the morphology and early evolution of the female cone in the conifers.

THE GENUS *Lebachia* Florin

The "genus" *Walchia,* first distinguished in 1825 by von Sternberg, contains at least two natural groups of species, one of which has now been named *"Lebachia"* after Lebach, a well-known Lower Permian plant locality in the Saar. *Lebachia* is the dominant genus of early conifers in the Northern Hemisphere. Fourteen species have been described; in addition, 11 species of similar kind are temporarily retained in the artificial genus *Walchia* because our knowledge is still too imperfect to allow their being referred to *Lebachia* with absolute certainty. Personally, however, I think that they, too, will later prove to belong to this natural genus.

The lebachias were monopodially branched woody plants of probably moderate height, built up of rather slender main stems and lateral, axillary, spreading shoot-systems forming regularly arranged but false whorls. In habit the lebachias obviously differed a good deal from the Cordaites which lacked the regular branching of the stem by uniform whorls. In young stages the stem is covered by bifacial, spirally disposed, leathery foliage leaves, broadly decurrent, pointing forward and overlapping. These leaves are linear-triangular in shape, two-forked at their apices, and probably uni-nerved in their basal and middle parts.

Each lateral shoot-system consists of a strong shoot of the first or penultimate order with radially distributed leaves. It carries two uniform rows of 20–60 mostly alternating lateral branchlets spread in the horizontal plane. The foliage leaves of the penultimate shoot resemble those of the stem but are sometimes entire at the apices (by reduction) and sometimes more spreading. In exceptional cases lateral shoots of the second or even third order have produced branchlets, thus making the system more complex (cf. recent araucarias of the Section Eutacta).

The branchlets are closely covered by homomorphic, overlapping, bifacial, spirally and radially arranged foliage leaves, pointing forward or more or less spreading. Further, they are concave adaxially, decurrent at their bases, of linear-triangular or almost linear shape, always entire at the apices, more strongly keeled on their backs than on their upper surfaces, and no doubt always uni-nerved. No bud-scales were formed.

The branchlet leaves are amphistomatic. The under side of such a leaf shows two short papillate groups or bands of stomata irregularly arranged but mostly orientated longitudinally. Its upper side has two long papillate stomatic bands, occasionally fused at the apex. They, too, are made up of irregularly arranged and as a rule longitudinally orientated stomata. The leaf margin is frequently denticulate.

The stomatal apparatus is of the haplocheilic type and is monocyclic or incompletely amphicyclic. The four to ten perigene subsidiary cells usually carry one cuticular papilla each; two of them are as a rule polar and all the rest lateral. Contiguous stomatal apparatuses are often found to have subsidiary cells in common. The guard-cells are sunk below the surface and only weakly cutinized. Cuticular papillae occur not only in the stomatic groups and bands but also outside these on the upper surface of the leaf, though in varying frequency and distribution. One-celled hairs are found on both sides, but they are generally more frequent and more closely placed on the under than on the upper surface. The epidermal cells have straight and smooth anticlinal walls.

The cones are of two kinds, female and male. The female cones are ellipsoid or cylindrical, more or less erect and seated singly and terminally, either on short branchlets in the basal parts of lateral shoot-systems or on the axes of penultimate order. They are built up of a main axis carrying spirally arranged, two-forked, non-lignified bracts, and in the axils of these, radially symmetrical or, exceptionally, slightly flattened fertile dwarf-shoots (seed-scale complexes or flowers). The bracts are amphistomatic, and their epidermal structure resembles that of the foliage leaves. The fertile dwarf-shoot has a short axis with several, broadly decurrent, erecto-patent, imbricate and spirally arranged appendages (scales), of which as a rule all but one are sterile and of narrowly triangular or almost linear shape. The fertile scale or megasporophyll is placed on the side facing the cone-axis, and carries one erect atropous ovule at its tip. This ovule is flattened and furnished with a single integument forming a direct continuation of the megasporophyll and completely enclosing the nucellus. Two archegonia were developed at the micropylar end of the female gametophyte. The integument is covered on the outer side by an epidermis with longitudinally orientated stomata in short rows or more irregularly arranged, as well as hairs and short cuticular papillae. The seeds are of the platyspermic type and furnished with a marginal "wing", in which there is an apical notch.

The male cones or flowers, placed terminally on leafy branchlets, are often more or less pendent, and ellipsoid or cylindrical. They consist of an axis with spirally arranged microsporophylls which are bifacial and spreading in the basal part but erect or erecto-patent in the distal region, and overlapping. Each microsporophyll appears to carry two microsporangia on the under side in the basal region. The epidermal structure of the microsporophyll resembles that of a foliage leaf. The pollen grains are of oval or almost circular outline in face view, but somewhat flattened at the poles. They are characterized by having a continuous balloon air-sac, interrupted only at the distal slightly sulciferous pole, and reticularly arranged mesexinous elements attached to the ectexine and protruding into the sac. No triradiate marking is visible.

Lebachia occurred exclusively in the Northern Hemisphere where it was widely distributed, particularly in Early Permian times. The following list gives the names of the species described up to now as well as a rough outline of their vertical and horizontal distribution. (Upper Carboniferous means "Stephanian"; Lower Permian, "Rotliegendes").

Lebachia americana Florin: Lower Permian of New Mexico and Texas.

L. angustifolia Florin: Lower Permian of the Saar, Thuringia and Moravia.

L. frondosa (Renault) Florin: Upper Carboniferous of Bohemia, France (Isère and Saône-et-Loire) and Portugal (?); Lower Permian of Thuringia, Saxony, Bohemia, Moravia, France (Aveyron and Saône-et-Loire) and England (Warwickshire, and Staffordshire ?).

L. garnettensis Florin: Pennsylvanian of Kansas; Lower Permian of Texas.

L. Goeppertiana Florin: Upper Carboniferous of France (Alsace and Isère); Lower Permian of South Norway, the Saar, Thuringia, Saxony, Bohemia, Moravia, France (Hérault and Saône-et-Loire), Portugal and Kansas.

L. Hirmeri Florin: Upper Carboniferous of Lower Silesia; Lower Permian of Lower Silesia, Bohemia and Moravia.

L. hypnoides (Brongn.) Florin: Lower Permian of Thuringia, Lower Silesia, Bohemia, Moravia, France (Hérault and Saône-et-Loire), Russia (Donetz) and North China (?).

L. intermedia Florin: Lower Permian of Bohemia and France (Hérault).

L. laxifolia Florin: Lower Permian of the Saar, Thuringia, Saxony, Lower Silesia, Bohemia, France (Hérault), Italy, Portugal and Morocco.

L. mitis Florin: Lower Permian of the Saar and Thuringia.

L. mucronata Florin: Lower Permian of the Saar and England (Staffordshire ?).

L. parvifolia Florin: Upper Carboniferous of the Saar, Bohemia, France (Loire and Saône-et-Loire), Portugal and England (Shropshire); Lower Permian of the Saar, Rheinpfalz, Wetterau, Thuringia, Harz, Saxony, Bohemia, Moravia, France (Hérault), Spain (Cantillana), Portugal, Morocco, East Greenland, Arizona (?), Kansas (?) and New Mexico.

L. piniformis (Schloth. pars) Florin: Upper Carboniferous of Lower Silesia and Eastern Canada (Prince Edward Island); Lower Permian of South Norway, the Saar, Rheinpfalz, Wetterau, Thuringia, Harz, Saxony, Lower Silesia, Bohemia, Moravia, Roumania, France (Hérault), Spain and England (Warwickshire).

L. speciosa Florin: Upper Carboniferous of France (Corrèze); Lower Permian of the Saar, Wetterau, Thuringia, Saxony, Bohemia and Moravia.

The species of the artificial genus *Walchia,* which strongly resemble *Lebachia* and will in all probability later turn out to belong to this genus, are:

Walchia Bertrandii Florin: Lower Permian of France (Hérault).

W. Carpentieri Florin: Lower Permian of Morocco.

W. Dawsoni D. White: Upper Carboniferous of Eastern Canada (Nova Scotia and Prince Edward Island); Lower Permian of Arizona.

W. gallica Florin: Lower Permian of France (Hérault).

W. Geinitzii Florin: Lower Permian of Italy.

W. minuta Florin: Lower Permian of Morocco.

W. Schlotheimii Brongn.: Lower Permian of France (Hérault and Saône-et-Loire) and Morocco (?).

W. Schneideri Zeiller: Pennsylvanian of Kansas; Lower Permian of France (Saône-et-Loire), England (Shropshire) and Morocco.

W. stephanensis Florin: Upper Carboniferous of France (Loire).

W. stricta Florin: Pennsylvanian of Colorado; Lower Permian of Russia (Perm) and Oklahoma.

W. Whitei Florin: Lower Permian of Texas and Arizona.

Isolated female cones which probably belong to *Lebachia* but are temporarily referred to a separate organ genus, *Walchiostrobus* Florin, have been found in the Lower Permian of Thuringia, Harz and France (Hérault and Vosges). Isolated male cones named *Walchianthus* Florin are known from the Lower Permian of Bohemia and France (Hérault), and stem fragments, provisionally referred to the organ genus *Walchiopremnon* Florin but probably belonging to *Lebachia,* from the Lower Permian of France (Vosges).

THE GENUS *Ernestiodendron* Florin

The artificial genus *Walchia* Sternb. has been found to include, besides *Lebachia,* also the genus *Ernestiodendron,* named after Ernst Friedrich von Schlotheim (1764–1832), a German geologist who in his "Petrefaktenkunde auf ihrem jetzigen Standpunkte" (Gotha, 1820) figured the specimens on which *Walchia* was founded.

Ernestiodendron comprises monopodially branched woody plants of probably moderate height, built up of rather slender stems and lateral, axillary, spreading shoot-systems forming regularly arranged but false whorls. In young stages the stem carries bifacial, spirally disposed, leathery foliage leaves which, in contrast to those of the lebachias, are non-decurrent and extremely spread out. In addition, these leaves are of linear-triangular shape and probably uni-nerved.

The lateral shoot-system consists of a strong shoot of the first or penultimate order with radially distributed foliage leaves. It carries two uniform rows of at least 50 mostly alternating lateral branchlets spread out in the horizontal plane. The leaves of the penultimate shoot resemble those of the stem. They fell off more easily than in *Lebachia,* leaving distinct scars on the axis.

The branchlets are covered by homomorphic, bifacial, spirally and radially arranged, rigid foliage leaves. These are non-decurrent, concave on their adaxial surfaces, linear-triangular in face view, acuminate at the apices and always entire, spreading or even pendent, and in all probability uni-nerved. Bud-scales do not occur.

The branchlet leaves are amphistomatic. Both surfaces show simple (or sometimes for a short distance double) longitudinal rows of stomata which are mostly orientated lengthwise but exceptionally crosswise. The rows of stomata are never grouped together into bands. In epidermal structure *Ernestiodendron* accordingly differs clearly from *Lebachia.* The leaf margin is denticulate also in *Ernestiodendron.*

The stomatal apparatus is of the haplocheilic type, and is monocyclic or incompletely amphicyclic. The perigene subsidiary cells usually carry one cuticular papilla each, and number from four to eight; two of them are as a rule polar, and all the rest lateral. Contiguous stomatal apparatuses with common subsidiary cells are rarely found. The guard-cells are sunk below the surface and only weakly cutinized. Cuticular papillae occur not only in the stomatic rows but also outside these in varying frequency and distribution. One-celled hairs are found on both surfaces but particularly on the under surface of the leaf. The epidermal cells have straight and smooth anticlinal walls.

The cones are of two kinds, female and male. The cylindrical female cones are seated singly and terminally on strong long shoots corresponding to the penultimate shoot of vegetative or male lateral shoot-systems. They are built up of a main axis carrying non-lignified, spreading bracts in a loose spiral and, axillary to these, flattened and more or less fan-shaped fertile dwarf-shoots (seed-scale complexes of flowers). The dwarf-shoot shows a few strong erect fertile scales or megasporophylls in the distal region, each carrying terminally one atropous erect or inverted (?) ovule with a single integument, which forms a direct continuation of the megasporophyll. The seeds were undoubtedly platyspermic.

The male cones or flowers are ellipsoid or cylindrical, mostly pendent and placed terminally on leafy branchlets. They consist of an axis with spirally arranged microsporophylls which are bifacial and spreading in the basal part but erect in the distal region and overlapping. Each microsporophyll appears to carry microsporangia on its under surface in the basal region. The pollen grains are ellipsoid or almost spherical but slightly flattened at the poles. They are furnished with a continuous balloon air-sac interrupted only at the distal, slightly sulciferous pole, and with reticularly arranged mesexinous elements attached to the ectexine and protruding into the sac.

Like *Lebachia, Ernestiodendron* occurred exclusively in the Northern Hemisphere, where it was widely distributed in Early Permian times. Only one species of this genus is hitherto known with certainty, but a few species of the artificial genus *Walchia* also in all probability belong to *Ernestiodendron,* although their position can not be definitely settled until better preserved material has been found and examined. Isolated female cones (*Walchiostrobus*) closely resembling those of *Ernestiodendron* are also known. The following list gives the names of all these forms and a rough outline of their vertical and horizontal distribution. (As before, Upper Carboniferous means "Stephanian"; Lower Permian, "Rotliegendes").

Ernestiodendron filiciforme (Schloth. pars) Florin: Upper Carboniferous of Moravia, France (Alsace and Isère) and Portugal; Lower Permian of South Norway, the Saar, Rheinhessen, Rheinpfalz, Thuringia, Saxony, Harz, Lower Silesia, Bohemia, Austria, Moravia, France (Corrèze, Hérault and Saône-et-Loire), England (Warwickshire), Russia (Orenburg), China (Shansi)?, Arizona and Texas.

Walchia Arnhardtii Florin: Lower Permian of South Norway, the Saar (?), Thuringia and France (Hérault).

W. germanica Florin: Lower Permian of Thuringia.

W. rigidula Florin: Lower Permian of Bohemia.

Walchiostrobus fasciculatus Florin: Lower Permian of Thuringia.

W. spp.: Lower Permian of Thuringia.

ARTIFICIAL GENERA CONTAINING MATERIAL OF *Lebachia* AND *Ernestiodendron*

Gomphostrobus Marion

Gomphostrobus has turned out to be simply a designation for bifacial leathery leaves occurring on Palaeozoic conifer shoots of various kinds. These leaves are linear-triangular or broadly triangular, two-forked isotomically at the apices, and no doubt uninerved in the basal and middle regions. The bundle bifurcates at the apex in order to supply the two lobes. These appear to have their narrow blades orientated at right angles to the main part of the leaf.

The *Gomphostrobus* type of leaf occurs on the stems, on the vegetative shoots of penultimate order and as bracts in the female cones of *Lebachia* and *Ernestiodendron*. It is also found in the genera *Palaeotaxites* and *Lecrosia* mentioned below. Finally, such leaves have been produced exceptionally on branchlets of Palaeozoic conifers, probably after wounding. The *Gomphostrobus* type is regarded as being either the juvenile form of leaf of Upper Carboniferous and Lower Permian conifers or—more probably—a transition form between their juvenile and adult leaves.

Tylodendron C. E. Weiss emend. Florin

This artificial genus is based on isolated portions of stem pith permeated by matrix and on casts of the medullary cavities of stems of walchias. They are relatively wide and have swellings at regular intervals corresponding to the regions where branches were given off in whorls. On the surface of such a fossil there are spirally arranged oblong-rhombic areas uniform in length or shortened at intervals. These areas are bounded by furrows produced by the primary vascular bundles of the stem. In the basal region of each area there is a median furrow corresponding to a leaf-trace. The pith consists of large thin-walled parenchyma cells. In longitudinal section it shows irregularly occurring transversal gaps between diaphragms of persistent tissue. The same structure has been found in the above-mentioned *Walchiopremnon.*

Fossils of this kind are known from the Upper Carboniferous of the Saar, France (Corrèze) and eastern Canada (Prince Edward Island) as well as from the Lower Permian of the Saar, Rheinpfalz, Wetterau, Saxony and Russia (Donetz).

Endolepis Schleiden

This "genus" comprises fossils similar to *Tylodendron* except that there are no swellings at intervals. On the surface they always show areas of equal length. They are of Palaeozoic and Early Mesozoic age. Although the branching of the stem is not indicated, part of the *Endolepis* fossils may have belonged to walchias. Nor were there any swellings at intervals in *Walchiopremnon* which certainly represents stem-fragments of this general type of early conifers.

Fossils referable to *Endolepis are* known from the Lower Permian of France (Corrèze) and Russia (Perm, Orenburg, and Donetz).

Dadoxylon Endlicher

This well-known general type of gymnosperm wood, found in *Walchiopremnon* and *Tylodendron,* was characteristic not only of the Palaeozoic Cordaites but no doubt also of the early conifers generally. It is impossible to decide which of the dadoxylons do belong to walchias and which do not, but at least two of the Lower Permian forms, *D. saxonicum* Goepp. emend. Frentzen and *D. Schrollianum* Goepp. emend. Frentzen, seem to represent conifers of this kind.

Cordaicarpus H. B. Geinitz and *Samaropsis* Goeppert

These artificial genera are based on isolated platyspermic seeds of Palaeozoic age preserved as compressions, and differ one from the other chiefly in the width of the marginal "wings" formed by the sarcotesta.

Of Upper Carboniferous and Lower Permian conifers, we know the seeds of only two species, *Lebachia piniformis* and *L. hypnoides*. They correspond to the *Cordaicarpus* type. Isolated seeds have occasionally been recorded from strata containing identifiable remains of *Lebachia* and *Ernestiodendron*. In most instances they belong to *Cordaicarpus*, but sometimes *Samaropsis* seeds have also been found. Considering the slight difference between these types, some of the latter may be seeds of species of early conifers.

Pollen-grains

Isolated pollen grains of the general type characterizing *Lebachia* and *Ernestiodendron* have been found in both Upper Carboniferous and Permian strata. They have been shown to be very similar to those of the Cordaites, and the same kind occurs in a conifer of Upper Permian age, probably referable to the genus *Ullmannia* Goeppert. Under these circumstances it does not seem possible to identify with any degree of accuracy the genus or even the family and class of such isolated pollen grains.

THE GENUS *Palaeotaxites* D. White

The genus *Palaeotaxites* comprises woody plants with copiously branched lateral shoot-systems made up of moderately strong axes of penultimate order with numerous branchlets apparently more or less spreading in all directions. The penultimate axis carries loosely arranged, bifacial, spirally disposed, triangular foliage leaves, bifurcated at their apices, while the branchlets are covered by entire leaves which are spirally arranged, overlapping, of linear-triangular shape, decurrent, curved inwards at their apices and in all probability uni-nerved. There are no bud-scales.

This genus resembles *Lebachia* but differs by the branchlets not being spread in one plane.

The single hitherto known species, *Palaeotaxites praecursor* D. White, occurs in the Lower Permian of Arizona (Grand Canyon).

THE GENUS *Lecrosia* Florin

Lecrosia comprises woody plants with irregularly (not pinnately) branched shoot-systems, each built up of a main axis of antepenultimate (or penultimate) order, carrying bifurcated foliage leaves and long, sometimes branched, lateral shoots spread approximately in one plane. When branched the lateral shoots carry a few short branchlets in the same plane. The branchlet leaves are arranged in rather close spirals, overlapping, more or less spreading in all directions. They are bifacial, decurrent, adaxially concave, almost linear or triangular-linear, entire, acute and in all probability uni-nerved.

Two species have been described, *Lecrosia Grand' Euryi* Florin and *L. Gouldii* Arnold. The former is of Upper Carboniferous (Stephanian) age and was discovered in France (Loire), while the latter belongs to the Lower Permian of Colorado.

THE GENUS *Carpentieria* Nemejc et Augusta

This genus is based on woody plants with lateral shoot-systems of regularly pinnate branching made up of a relatively weak penultimate axis and numerous branchlets in two rows spread in the horizontal plane. The leaves of the penultimate shoots are sparsely arranged, bifacial, spreading, decurrent, linear-cuneate in their basal halves and bifurcated at the apices. The branchlets have more closely arranged, spirally disposed, more or less spreading and overlapping bifacial leaves. These, too, are linear-cuneate in their basal halves and bifurcated at their apices. Each is apparently traversed by a median bundle bifurcating in the apical region.

The leaves of *Carpentieria* are thus exclusively two-forked, while *Lebachia* has bifurcated leaves on its stems and branches but entire leaves on its branchlets. In addition, the bifurcated leaves of the two differ somewhat in shape.

Two species of *Carpentieria* are known, *C. marocana* Nemejc et Augusta and *C. frondosa* (Goepp.) Florin, both of Lower Permian age. The former occurs in Moravia, the latter in Bohemia and Morocco.

THE GENUS *Buriadia* Seward et Sahni

The genus *Buriadia* is based on woody plant-remains consisting of lateral, sparsely and irregularly branched shoot-systems with strong penultimate axes and scattered, non-parallel, more or less spreading branchlets, not distinctly arranged in one plane. The penultimate axes have scattered, spirally disposed, bifacial foliage leaves pointing in all directions. These are straight or, on the adaxial side, more or less convex, decurrent, linear-cuneate or cuneate, as a rule repeatedly forked at the apices and traversed by dichotomizing bundles. The branchlets, too, have spirally arranged, bifacial, decurrent leaves, spreading more or less in all directions, but these are linear or linear-cuneate and bifurcated at the apices; each is traversed by a bundle which is dichotomized only once.

The leaf-epidermis is characterized by monocyclic or incompletely amphicyclic stomata of the haplocheilic type with a moderate number of subsidiary cells. The epidermal cells are often papillate, and their anticlinal walls straight and smooth.

Only one species, *Buriadia heterophylla* Seward et Sahni, is known so far, and this occurs in the Lower Gondwana formation of India (Karharbári beds) and the Upper Carboniferous (Tubarão-Series) of Brazil [Rio Grande do Sul, Santa Catharina (?), and Paraná (?)].

THE GENUS *Paranocladus* Florin

This is a genus of woody plants with irregularly branched (not pinnate) lateral shoot-systems consisting of scattered branchlets on axes of penultimate order. The branchlets are covered by homomorphic spirally arranged leaves, either somewhat spreading or adpressed, which are entire, bifacial, broadly decurrent, triangular-linear and probably uni-nerved.

The branchlet leaves are amphistomatic. On the under side there are two slightly papillate groups of scattered irregularly arranged but mostly longitudinally orientated stomata. On the adaxial side, on the other hand, there are two long stomatic bands containing scattered and irregularly distributed but mostly longitudinally orientated more strongly papillate stomatal apparatuses. The leaf-margin has small scattered teeth in the basal region of the leaf.

The stomatal apparatus is of the haplocheilic type, monocyclic or incompletely amphicyclic. It has five to seven perigene subsidiary cells—two or which are polar—each furnished with a cuticular papilla on the upper sides of the leaves. The guard-cells are sunk below the surface and weakly cutinized. Outside the stomatal apparatuses, cuticular papillae occur only on the upper surfaces of the leaves. There are no hairs at all. The anticlinal walls of the epidermal cells are straight and smooth.

Two species have been described. *Paranocladus Dusenii* Florin has been found in the Permo-Carboniferous of Brazil, State of Paraná (Passa Dois Series). *Paranocladus* (?) *fallax* Florin occurs in the Upper Carboniferous of the same State and probably also in the Permo-Carboniferous of Argentina (San Luis and La Rioja).

THE VEGETATIVE ORGANS OF THE UPPER CARBONIFEROUS AND LOWER PERMIAN CONIFERS

In one case the top of a stem of *Lebachia piniformis* has been found, preserved as an impression. It has a whorl of five lateral, axillary, extremely spreading shoot-systems below a thick bud-like body. This body consists of the primordia of the next whorl of branches, protected by an envelope of two-forked leaves of the *Gomphostrobus* type. Since the leaves on the stem are spirally arranged, however, the branches are not placed at exactly the same level; they form a false whorl. The same conditions have been demonstrated in other species of *Lebachia*. Older stems often show at intervals swellings which apparently correspond to the zones from which the whorls of branches arose. All these are monopodially branched woody plants characterized by a regular arrangement of the branch whorls. The number of branches in each whorl is five or six, which corresponds to the living *Araucaria excelsa* R. Br., the Norfolk Island pine. In *Lebachia piniformis* a few short and weak unbranched lateral shoots sometimes occur between consecutive branch whorls, indicating that the stem has had more axillary buds than suggested by the whorled branching itself. These weak lateral shoots outside the whorls are irregularly arranged and seem to have been shed early. The whorled branching of the stem is also characteristic of *Ernestiodendron,* but in this genus no traces of weaker shoots outside the pentamerous whorls have been observed.

The monopodial construction of the stem and its whorled branching indicate that these conifers were trees. Their stems were apparently rather slender and of moderate height, although there may have been differences in this respect in the several species, judging by the dimensions of their lateral branches.

Hardly anything is known of the growth forms and types of branching of the remaining Palaeozoic genera of conifers. *Buriadia, Lecrosia* and *Paranocladus,* however, would seem to have been less strongly specialized in this respect than *Lebachia* and *Ernestiodendron.*

Two main types of lateral shoot-systems can be distinguished among the conifers of the Upper Carboniferous and Lower Permian, viz., the *Walchia* and *Buriadia* types. The *Walchia* type corresponds to the very marked form of lateral shoot-system characteristic of *Araucaria excelsa.* It consists of a shoot of the first or penultimate order carrying relatively small radially distributed leaves but always two rows of branchlets spread in one plane. In *Lebachia* and *Ernestiodendron* the lateral shoots of the second order usually remained unbranched. The number of branchlets varied from 20 to 60, whereas the maximum number in *Araucaria excelsa* is 120.

In a few cases lateral shoots of the second order have in their turn produced branchlets arranged in the same manner, thus making the shoot-system more complex. Very occasionally these shoots, too, have behaved similarly but have then given off branchlets only on the side facing the apex of the whole system.

Besides the whorled branching of the stem, the regular construction of the lateral shoot-systems indicates that *Lebachia* and *Ernestiodendron* closely resembled *Araucaria excelsa* in habit. In this living conifer the lateral shoot-systems gradually become more complex with age, the shoots of the second order producing lateral branchlets arranged in two rows. The said fossil conifers seem to have been similar in this respect.

In contrast to the *Walchia* type, the mode of branching of the *Buriadia* type is less strongly specialized. The branchlets are not arranged in two rows but more irregularly and sparsely, although the leaves are disposed all round the axes. This refers to the genera *Buriadia, Paranocladus* and *Lecrosia.*

The *Buriadia* type appears to be relatively primitive, and the *Walchia* type consequently specialized. The profuse development of the latter in the Northern Hemisphere in Late Palaeozoic times is very remarkable. In the South it did not occur at all at that time. But in the middle of the Permian period other types of branching suddenly replaced the *Walchia* type which was then confined to the Mesozoic and Caenozoic *Araucaria,* a genus now represented exclusively in the Southern Hemisphere.

Apart from cotyledons and true juvenile leaves which, of course, are never preserved, two more or less specialized categories of foliage leaves can be distinguished in the Upper Carboniferous and Lower Permian conifers: *a*) leaves on the main axis and on lateral axes of the first order (i.e., the penultimate or antepenultimate order), which are frequently bifurcate; and *b*) entire leaves on lateral axes of the ultimate or sometimes penultimate order.

The leaves of the first category are longer, broader and somewhat more flattened than those of the second. They are uninerved and needle-like, spirally disposed and pointing in all directions, almost straight or more or less curved. They are broadly decurrent in *Lebachia,* but non-decurrent in *Ernestiodendron.* They are further triangular or almost linear, either entire and acute at the apices or frequently two-forked, in which case the bundles are also divided by dichotomy into two branches in the apical part. Finally the leaves of *Lebachia* are turned outwards only slightly, while those of *Ernestiodendron* are extremely spread out. In *Lebachia* the leaves persisted for a long time as they do in the living araucarias. In *Ernestiodendron* the leaves left distinct scars on the axis and fell off more easily than in *Lebachia.* The two-forked leaves are of a type quite unknown in living conifers. The blades of the lobes were apparently perpendicular to the main part of the leaf, and the forking accordingly in the nature of cruciate dichotomy.

Lebachia and *Ernestiodendron* differ distinctly from the araucarias in the number of leaf-traces entering each leaf and in the venation of the leaves. Both lack true bud-scales, however, and the shoot-tips are protected by ordinary or only slightly arrested leaves.

The foliage leaves of the second category in *Lebachia* and *Ernestiodendron,* which are attached to the lateral axes of ultimate order, are needle-like to scale-like, spirally arranged, imbricate and pointing in all directions. They are more or less curved, less spreading in *Lebachia* than in *Ernestiodendron,* and decurrent in the former genus in contradistinction to the latter. Finally they are triangular to almost linear, always entire at the apices and no doubt uni-nerved. These branchlet leaves differ from the leaves on the stem and lateral branches of the first order in that they are always entire and smaller.

Several of the earliest conifers were thus characterized by heterophylly. The two-forked leaves may be assumed to represent an intermediate stage between the juvenile and the adult leaf-forms.

The peculiar leaf-forms of *Carpentieria* and *Buriadia* are of great interest in this connection. The taxonomic position of these genera certainly can not be conclusively established in the absence of reproductive organs, but the appearance of the lateral shoot-systems points to their being related to the walchias and other Palaeozoic conifers. In some of the living conifers, e.g., the common juniper (*Juniper communis*), the juvenile or an only slightly changed leaf-form is retained in the adult stage as a feature characteristic ot the species; and in other Cupressaceae the juvenile and transitional forms may be fixed and propagated by certain methods. It appears plausible to regard the invariably dichotomized leaves of *Carpentieria* from this point of view. At all events, *Carpentieria* gives the impression that in Palaeozoic conifers the dichotomized leaf has preceded the entire leaf and is more primitive. The entire leaf appears in other words to have arisen by reduction.

The leaves of *Buriadia* differ still more from those of *Lebachia* and *Ernestiodendron.* The blades and bundles dichotomize once or several times, although the branchlets appear always to carry only two-forked leaves. We have here essentially the same phenomenon as in the lebachias; the last developed leaves are arrested in their development and have more simple form and venation. The conifers of the Upper Permian and later periods have no such dichotomizing leaves.

All hitherto known Palaeozoic conifers differ sharply from the Cordaites in leaf-morphology. This applies not only to the size, form, venation and arrangement of the leaves but also to the vascular supply. Among living conifers, however, the general appearance of the broad pluri-nerved leaves of the genus *Agathis* and of the section Nageia of *Podocarpus* is rather like that of the Cordaites.

The anatomy of the vegetative organs of the Upper Carboniferous and Lower Permian conifers is still very imperfectly known. As in the higher gymnosperms generally, the stem contains a true eustele with endarch protoxylem. But—in contradistinction to the Cordaites—the primary wood is poorly developed. The secondary wood sometimes has feebly marked growth rings resembling those of the living araucarias.

As in Cordaites and the araucarias, the secondary wood is characterized by being composed of tracheids of the araucarioid type. Wood parenchyma does not occur at all or only sporadically, nor are there any resin canals. As in the living araucarias but in contrast to Cordaites, the tracheids of the walchias have only one to three rows of closely arranged alternating bordered pits in their radial walls. The wood-rays are usually uniseriate, as in the living araucarias. The pith is relatively thick and becomes irregularly ruptured in elongation of the stem, but not in the same way as in the Cordaites.

The internal structure of the *Lebachia* and *Ernestiodendron* leaves is quite unknown, but it has been possible to study their epidermal structure, owing to the extraordinary resistance of the cutinized layers. As regards external morphology and epidermal structure, four types of foliage leaves may be distinguished, of which the *Lebachia* and *Ernestiodendron* types are the best known.

In both these types the leaves are amphistomatic and their margins often denticulate. But they differ in the arrangement of the stomata. In the *Lebachia* type there are on the under side of the leaf two separate papillate stomatic groups or bands containing more or less irregularly distributed but mostly longitudinally orientated stomatal apparatuses. On the upper side there are two stomatic bands of the same kind, but these run from the base almost to the apex of the leaf. The stomatal apparatus is of the haplocheilic type and is monocyclic or amphicyclic. There are from four to ten subsidiary cells, each usually provided with a cuticular papilla. Subsidiary cells are often common to contiguous stomatal apparatuses. On the upper side of the leaf there are cuticular papillae also in the non-stomatiferous areas. In addition, the leaves have uni-cellular hairs on both sides, more particularly on the under.

The *Ernestiodendron* type, on the other hand, is characterized by more or less widely separated isolated rows of stomatal apparatuses, mostly orientated lengthwise, on both sides of the leaf, and has accordingly no stomatic bands. Here, too, the stomatal apparatus is haplocheilic as well as monocyclic or incompletely amphicyclic, and has four to eight mostly papillate subsidiary cells. But in *Ernestiodendron* these cells are rarely common to contiguous apparatuses. Cuticular papillae also occur outside the stomatic rows, and there are unicellular hairs on both sides of the leaves.

Ernestiodendron differs from all other known conifers of Upper Carboniferous or Lower Permian age by the arrangement of the stomata in isolated rows. This genus is as markedly different from *Lebachia* as for instance the Eutacta section of *Araucaria* is from the genus *Cryptomeria* among living conifers. On the other hand, its epidermal structure resembles that of the Cordaites as well as of certain Upper Permian conifers.

If finally we compare the leaf-epidermis of the earliest conifers with that of living forms it will be seen that none of the living species groups presents the same combinations of the characteristics as are found in the fossils, although the principal features are similar. In contrast to certain living conifers, the Palaeozoic forms seem completely to lack calcium oxalate in their cuticular layers, and *Lebachia* and *Ernestiodendron* differ from both the contemporaneous Cordaites and the geologically younger conifers by the hairiness—particularly on the under sides—of their leaves. It is true that hair-like epidermal cells may in a few cases be found in living conifers, but they are confined to the leaf-margins or may sometimes occur also on galls or leaves protecting buds. Hair-like structures also develop in the specialized stomatal bands of the living torreyas, but they differ essentially in type and distribution from the simple uni-celled hairs of *Lebachia* and *Ernestiodendron*. The hairiness of their foliage leaves is thus giving these ancient genera a unique position among the conifers.

REPRODUCTIVE ORGANS OF THE UPPER CARBONIFEROUS AND LOWER PERMIAN CONIFERS

The oldest conifers were probably monoecious, but the male and female cones were borne on separate shoot-systems. In one single case cones of both sexes have been found on the same shoot-system of *Lebachia piniformis.* This points to the lebachias as being monoecious. Monoecism with unisexual cones has also been found in the Cordaites. This type of sexual differentiation therefore appears to have been primary in that division of the gymnosperm complex which comprises the closely related conifers and Cordaites.

The female cones of the oldest conifers are placed terminally on lateral axes of the ultimate or penultimate order. In *Lebachia piniformis* they are always found at the end of leafy branchlets which as a rule belong to the basal region of the shoot-system, while typical vegetative branchlets are developed above them. The fertile and sterile regions are frequently separated, but a vegetative branchlet may occasionally be seated between two female cones, or a few such branchlets beneath the female cones. On the other hand, the vegetative lateral branchlets of certain lebachias are replaced by cone-bearing shoots all along the main axis of the lateral system. The basal branchlets, terminated by female cones, are often shorter and stronger than the vegetative and male branchlets. The female cones are more or less upright, but the male flowers are pendulous. The shoot-systems carrying exclusively female cones in the lebachias were apparently rather like the corresponding systems in the living *Araucaria* species of the section Eutacta, for instance, *Araucaria columnaris,* although their shoots and cones were of different dimensions.

Most of the lebachias seem to have borne their female cones terminally on axes of the last order. But at least *Lebachia hypnoides* and one other species bore them terminally on lateral axes of the penultimate order. Contrary to the branchlets, the branches of the penultimate order are covered with two-forked leaves. Strong fertile branches have sometimes several vegetative branchlets below the cone, but others have no such branchlets and appear to carry the female cone at the end of a long leafy stalk. From this we may conclude that *Lebachia hypnoides*—having on the one hand profusely branched vegetative and male shoot-systems and but slightly branched female systems on the other—was somewhat more differentiated than *Lebachia piniformis.*

While the lebachias were thus differentiated into two types in respect to the position of the female cone, viz., one primitive, in which the cones were placed singly and terminally on leafy branchlets, and one more advanced, in which they were seated singly and terminally on penultimate shoots with few or no branches, the latter type alone is represented in *Ernestiodendron,* the other dominating conifer genus of the Late Carboniferous and the Early Permian.

In contradistinction to the female cones, the male flowers of *Lebachia* and *Ernestiodendron* are always placed terminally on leafy branchlets. They are either confined to the basal region of the system, as in *Lebachia piniformis,* or occur along the whole penultimate axis, as in *Lebachia hypnoides* and *Ernestiodendron filiciforme.* In *Lebachia piniformis* the female cones and male flowers are thus similarly placed on the lateral shoot-systems, while in the other conifers mentioned above their position is different. The male flowers are usually more or less pendulous, while the female cones are upright.

Lebachia and *Ernestiodendron* differ sharply from the Cordaites in the arrangement of the male flowers but correspond to certain geologically younger genera as well as to some living conifers. The male conifer flowers of Palaeozoic times are never placed directly in the axils of leaves or bracts on penultimate axes, as they generally are in living representatives of the class, nor are they grouped into any kind of inflorescences.

As far as we know at present, two main types of female cones can be distinguished in the earliest conifers: the *Lebachia* type and the *Ernestiodendron* type. The *Lebachia* type is characteristic of *Lebachia* and several of the walchias that probably belong to the same genus. The *Ernestiodendron* type occurs in *Ernestiodendron* and in a few walchias which are very likely related to this genus. The female cone of *Lebachia* is ellipsoid or cylindrical and compact. Its main axis carries numerous spirally arranged non-lignified two-forked bracts, the bundles of which dichotomize and send one branch into each of the two lobes. In the axil of each bract there is a radially symmetrical dwarf-shoot, i.e., a seed-scale complex or flower made up of a short lateral axis with a varying number of spirally disposed and broadly decurrent upturned scales. As a rule all but one of these scales are entire and sterile. The fertile scale faces the main axis of the cone and terminates in a single erect ovule. This fertile scale—the seed-scale or megasporophyll—is in the same position on the floral axis as the sterile scales, as all scales are arranged together in one single spiral. A few sterile scales are seated between the megasporophyll and the base of the dwarf-shoot. The sterile scales diminish in length and breadth towards the base and the apex of the dwarf-shoot.

The ovule, which is apparently slightly flattened like the megasporophyll, has a single integument which is a direct continuation of the sporophyll, and a relatively deep micropyle. In contradistinction to the sterile scales, the fertile scale is thus forked in the apical region.

Although the female dwarf-shoot of *Lebachia* is essentially symmetrical radially, the position of the sporophyll in this type, with its ovule on the side facing the cone axis, is an early though slight indication of dorsiventrality.

Lebachia Goeppertiana appears to have more numerous sterile scales on the flower axis than *L. piniformis,* but the base of its megasporophyll is not covered by such scales. The flower of *L. hypnoides* resembles that of *L. Goeppertiana,* but is more flattened and has fewer sterile scales. *Lebachia hypnoides* is more advanced, as far as the flattening of the flower is concerned, than any other known species of this genus, but the scales are apparently still spirally arranged on the axis and the course of the bundles has probably not been much influenced.

Occasionally the *Lebachia* cones proliferated, just as the cones of, for instance, living larches as well as of *Cryptomeria* and *Cunninghamia* do, thus changing from the reproductive to the vegetative phase.

In *Ernestiodendron* the female cones are cylindrical but less compact than the *Lebachia* cones. Their main axes carry numerous spirally arranged, non-lignified, and more or less spreading bracts, probably always two-forked at the apex. The *Ernestiodendron* bracts agree in appearance and venation with those of *Lebachia.*

A fertile more or less flattened and in general appearance somewhat fanshaped dwarf-shoot was developed from the axil of each bract. This seed-scale complex or flower may be shorter, as long as, or longer than the bract. Each flower here has three to five sporophylls but apparently no sterile scales at all. The *Ernestio-*

dendron flowers accordingly differ considerably in appearance from those of *Lebachia,* but their morphological connection is evident from the organization of two flowers of the *Ernestiodendron* type, which—though found isolated—in all probability belong to this genus. Notwithstanding the rather strong flattening of the flower, its axis still carries 20 to 30 scales, of which four to six are megasporophylls with terminal ovules, and all the rest sterile. There can hardly be any doubt that these scales occupy the same positions as in *Lebachia,* i.e., the sterile and fertile scales are arranged in one simple spiral. The ovules are erect in one of the flowers but appear to be inverted in the other. In *Walchia germanica,* which is likewise probably referable to *Ernestiodendron,* the sterile scales have disappeared almost completely, only one such scale being sometimes found at the base, while in the apical region there are three to seven megasporophylls with inverted ovules. In the *Ernestiodendron* type, too, the integument is the direct continuation of the sporophyll, and proliferated cones occur as in *Lebachia.*

The study of the female cones of the earliest conifers has thus disclosed morphological conditions of extraordinary interest. First it may be inferred that the compound strobilus is the primary form of female reproductive organ in the conifers, i.e., bracts and seed-scale complexes had very early united into a more or less compact cone. If the flower is defined as a sporophyll-carrying shoot of limited growth, the axillary dwarf-shoot or seed-scale complexes of the earliest conifers and the Cordaites may be called flowers, and associations of such flowers inflorescences. The seed-scales might be designated megasporophylls, but only if we discard the old and now untenable conception that the sporophylls were derived from foliage leaves.

The material now examined varies a good deal in respect to the formation of the seed-scale complexes. To sum up, one may say that the *Lebachia* type is characterized by radially or almost radially symmetrical, fertile dwarf-shoots, either unflattened or slightly flattened, which, while carrying several to numerous sterile scales, usually have only a single seed-scale in the basal or middle region of the axis. The sporophylls have the same position on the axis as the sterile scales, i.e., all scales are arranged consecutively along a simple ascending spiral. The ovules are placed singly and terminally on the sporophylls, and are always erect. In contradistinction to this type, *Ernestiodendron* has more or less flattened dwarf-shoots, bearing only a few sterile scales—or even none at all—at the base and three to seven megasporophylls in the middle or apical region of the axis. Here, too, the ovules are placed singly and terminally on the sporophylls, but they are either erect, as in *Lebachia,* or inverted. In the *Lebachia* type the dwarf-shoots still show the presence of an axis rather distinctly, but in *Ernestiodendron* the axis is more strongly reduced and more concealed. *Lebachia* represents the more primitive type of the two, barring the reduction of the number of seed-scales. *Ernestiodendron* is the more advanced type in several respects, viz., with regard to the symmetry of the dwarf-shoots, reduction of the flower axis and the number of sterile scales on this, and the recurvation of the sporophyll tips resulting in the inversion of the ovules. In further contrast to the *Lebachia* type, the *Ernestiodendron* type varies rather widely in the formation of the seed-scale complexes. But these complexes are evidently homologous everywhere, and this also applies to the seed-scales. The uniform appearance of the bracts, probably always two-forked at their apices, contrasts with the variability of the seed-scale complexes arising from their axils.

The investigation into the morphology of the seed-scale complexes of Palaeozoic Cordaites and conifers has given the clue to the interpretation of the female cones of Mesozoic and Caenozoic conifers. Disregarding such changes as the shortening of the cone-axis internodes and the reduction in number of its lateral organs, the great morphological diversity in the female sex of later conifers is chiefly due to the transformation and reduction in various directions of a primitive fertile dwarf-shoot and its bract.

The ovules of the earliest conifers are bilaterally symmetrical. They are always atropous but sometimes inverted. They have a single integument which forms a direct continuation of the megasporophyll. As in the Cordaites, that integument appears to be formed out of two transversely placed primordia, united by the growth of the underlying tissues—and the same applies to several, if not all, living true conifers.

Unfortunately we know very little about the internal structure of the ovule. The nucellus, however, is entirely enveloped in the integument, apparently free from it except in the chalazal region. The tip of the nucellus consists of large inflated cells and probably served to facilitate penetration by the pollen-tubes. The gametophyte seems to have produced two archegonia at its micropylar end.

Turning now to the male flowers of the earliest conifers, these are radially symmetrical, cylindrical or ellipsoid. They consist of an axis and numerous entire, bifacial and dorsiventral microsporophylls, arranged in a close spiral and imbricate. These sporophylls have a basal, narrow, occasionally stalk-like, spreading portion, and a distal leaf-like, up-turned portion. They are hyposporangiate and bisporangiate, i.e., the two microsporangia are seated on the under side of the basal part of the sporophyll. There are no sterile scales at all above the basal region of the flower. The male and female reproductive organs thus differ considerably, which is particularly conspicuous in the lebachias where the two kinds mostly correspond to each other in position on the shoot-systems. In the one case we have simple flowers, in the other inflorescences. The microsporophyll is hypopeltate, i.e., furnished on its under side with an outgrowth protecting the sporangia.

The male reproductive organs of the earliest conifers and the partly contemporaneous Cordaites differ greatly. In both groups the male flowers certainly have an axis with radially and spirally arranged lateral scales, but in the conifers that axis carries exclusively hypopeltate, hyposporangiate and bisporangiate microsporophylls, whereas in the Cordaites the microsporophylls are intermixed with sterile scales on the axis and of a simpler shape, except in the apical region where they form into clusters of four to six terminal microsporangia. The male flowers of the Cordaites are moreover assembled into inflorescences, while those of the Upper Carboniferous and Lower Permian conifers are always seated singly and terminally on lateral branchlets. The microsporophylls of the Cordaites differ sharply from their foliage leaves. In the partly contemporaneous conifers the vegetative leaves are gradually replaced by microsporophylls of much the same size and general shape. The same applies to several Upper Permian and geologically still younger conifers which likewise have hypopeltate and hyposporangiate microsporophylls, even though the sporangia vary a good deal in number. The male flowers of the lebachias resemble those of the living Pinaceae, Podocarpaceae and some Taxodiaceae, although they do not correspond completely to any of these types. In the number of sporangia the lebachias agree with the living Pinaceae, but they also resemble the Araucariaceae in having free sporangia.

Lebachia and *Ernestiodendron* have pollen grains of the same general type as the Cordaites. These are characterized by a continuous balloon air-sac interrupted only at the distal pole, by reticularly arranged mesexinous elements attached to the ectexine and protruding into the sac, and by a shallow germinal furrow at the distal pole. The earliest conifers were evidently—like the Cordaites—wind-pollinated.

The pollen grains of the Upper Permian conifers are of the same or a similar kind. From the Early Mesozoic onwards, including the present time, conifer pollen grains are known with as well as without air-sacs. The study of the oldest conifers of the Northern Hemisphere has thus shown that pollen grains with a single balloon air-sac and a distal germinal furrow are primitive in this class of gymnosperms. The reduction of the all-over balloon sac into two bladders by the suppression of its proximal part, i.e., the change of the *Lebachia* grain to the modern type, appears to have been in some way associated with the inversion of the ovules, which took place in late Palaeozoic times. The Permian two-winged grains in their turn lead directly to those of certain living Pinaceae and Podocarpaceae.

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ECOLOGY OF THE GRASSLAND. II.[1]

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INTRODUCTION

The first paper under this title was restricted largely to a discussion of the characteristics of the composition (floristics and structure) of plant communities and methods of measuring them, and the present article will be similarly limited. In comparing the period since 1938 with earlier years it is noted that but few procedures have been developed during the later period, and that greater attention has been given to the testing and comparison of methods which had been devised in the previous 30 years, and to the refinement of these methods. Since 1938 it has also been realized that more attention should be given to the various characteristics of community composition. This increased emphasis on the study of a variety of characteristics and the careful selection of methods now used in acquiring data are due to refinements in the work of grassland ecologists and to the great expansion of research by agronomists and others which followed recognition of the importance of grasslands, not only in grazing but also in controlling erosion and in maintaining and building soil fertility. Advances have been made also toward standardization. Studies by different investigators of certain characteristics by the same method have facilitated comparison of descriptions of communities, examples of which will be shown. Even though most ecologists might not agree upon nor accept any standardized set of characteristics or methods of measurement, an agreement which perhaps is not warranted by the present state of development of grassland ecology, many ecologists might be willing to include certain characteristics and methods as part of their investigation in order to make their data comparable to those collected by others. One of the purposes of this paper is to present, on the basis largely of much of the research accomplished in grassland ecology in the decade since 1938, some of the advantages, with examples, of studying clearly specified community composition characteristics by certain methods, and to facilitate selection of characteristics and methods to use in particular investigations.

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CLASSIFICATION OF CHARACTERISTICS OF THE COMMUNITY

The classification of the characteristics of the community which was presented in the earlier paper on this subject and which has proven useful in planning research and in analyzing publications in this field is repeated below with some slight modifications. It is based to a large extent upon the work of Rübel (227, 228), Braun-Blanquet (18), Salisbury (229) and Nichols (183):

I. Composition of the community (Gesellschaftsgefüge, Zusammensetzung), including floristics and structure (organization) as well as the resulting physiognomy.

   A. Analytical characteristics.

      a. Qualitative characteristics.

         1. Kinds of species occurring in the community (floristic composition).
         2. Stratification of organisms or their parts above and below the soil surface.
         3. Periodicity (phenology, aspectation).
         4. Vitality (vigor of individuals of a species, Gedeihen).
         5. Life-forms (including vegetation forms, growth forms, habitat forms).
         6. Sociability (gregariousness) and association of species.

      b. Quantitative characteristics.

         1. Number of individuals (abundance, population density).
         2. Cover (area occupied, Deckungsgrad).
         3. Height of plants.
         4. Weight of plants (yield, production).
         5. Volume occupied by organisms.
         6. Frequency.

- B. Synthetic characteristics.
  1. Presence and constancy.
  2. Fidelity (Gesellschaftstreue).
  3. Dominance.
- II. Environmental relations of communities.
- III. Geographic distribution of communities.
- IV. Plant succession and processes involved.
- V. Classification of communities.

The above classification shows some changes in wording as compared to the 1938 classification in order to express the concept more clearly or to make it conform to the best current usage. "Habitat forms" has been included under "life forms" instead of placing it in a separate category. "Association of species" has been added to "sociability" in view of recent work that shows its importance. "Density" is omitted as a synthetic characteristic, although there are valid reasons for considering it in this class, but it is used under "number of individuals," in conformance with the best current usage. Categories II to V have not been subdivided because they are not treated in detail in this paper. A complete description of a community must necessarily deal with each of these characteristics, as well as with the first category. The characteristics under "composition of the community" will be discussed under "nature of the characteristic", methods of studying each, particularly as to refinements or developments of new methods, relationships to other characteristics, values and importance of the data and conclusions. Sufficient references will be cited and examples given to clarify concepts, to illustrate methods and to indicate the growth of this phase of grassland ecology. There is considerable justification for Conard's (54) statement that "No method of examination and description of vegetation has been so productive and suggestive as that of the recent plant sociologists".

DELIMITATION OF THE COMMUNITY

Two common approaches to ecology are the "habitat ecology" approach, in which the habitat is studied as an environment suitable for certain organisms, and the "community ecology" approach, in which the association of organisms is emphasized (81). The community approach has been considered from two viewpoints: one, as illustrated especially by Clements (42, 46, 47), in which emphasis is placed upon succession; the other, where the chief emphasis is placed upon composition, i.e., the kinds and relationships of the organisms within individual communities. These two are largely a matter of emphasis because neither viewpoint excludes consideration of the other. Clements worked out a system of classification, based upon succession, from the smallest communities (clans, families, locies) to the six large associations comprising the grassland formation of North America. Stebler and Schröter (249), in analyzing the meadows of Switzerland, were early leaders in studying the composition of small communities. Conard (53) recognized 71 plant associations in the central part of Long Island. Ostvald (189), on the basis of floristic composition, classified 40 square miles of moorland into 164 separate associations. These two viewpoints have been discussed in a number of papers (54, 27, 29, 105, 35, 36, 38). The community approach may include animals as essential parts of the community so that the whole aggregation of plants and animals is regarded as a biome or complex organism (48, 36, 38, 39, 86, 126); or plants and animals may be looked upon as forming distinct communities, or the plants as forming the community and the animals as being dependent upon them.

Delimitation of plant communities is complicated by the great diversity of grasslands, both artificial and natural, such as short-grass plains, tussock prairies, mowed meadows, alpine grasslands, irrigated and other tame pastures, lawns and short-term leys. The natural grasslands are being modified at an accelerated rate by grazing, mowing, fertilizer treatments, spraying or dusting to eliminate undesirable species, by the seeding of introduced species (especially noteworthy is the widespread seeding of crested wheatgrass, *Agropyron cristatum*, and other species in western United States), by the seeding of selected strains of native species and by conservation methods to reduce run-off and erosion.

The concept, the terminology and the bases for delimitation of plant communities have received considerable attention during the past 20 years. Due to differences in the viewpoints of investigators, to the different kinds of vegetation in which they have worked, and to differences in the objectives, requirements and facilities of their investigations, a variety of results and generalizations about the communities has resulted. This may appear somewhat confusing, but actually the condition is healthy because in the end it is highly probable that the soundest procedures and concepts will prevail, provided that the state of flux continues until all contributory elements have been included before a hardening into rigid rules and fixed concepts takes place.

Gleason (105) described three viewpoints regarding the nature of the plant association: *a*) the plant association is an organism, or a pseudo-organism, of individual plants and animals, held together by close bonds of interdependence and having properties and structural features similar to an individual organism; *b*) the plant association is not an organism but a series of separate units, variable in size but repeated in numerous examples, comparable to a species which is composed of variable individuals; the association is considered by some as a concrete entity merely divided into separate entities; by others as a mental concept, based on common characters of all of its separate parts and capable of typification by one or more of the pieces which most nearly approach the average or ideal condition; *c*) the individualistic concept, according to which the vegetation unit is a temporary and fluctuating phenomenon, dependent in its origin, its structure and its disappearance on the selective action of the environment and on the nature of the surrounding vegetation; it has no similarity to an organism and is scarcely comparable to a species; this individualistic concept of the plant community was first presented by Gleason in 1917.

Four features are fundamental, according to Gleason (105), in the delimitation of a plant community: *a*) homogeneity of structure; *b*) area (which may be large); *c*) definite limits to the area occupied; and *d*) duration in time. "The community is a complex or mosaic of slight irregularities, all of which blend into an entirety of apparent homogeneity. . . . A community is uniform, either in space or in time, only to a reasonable degree. This uniformity is sufficient to enable us to recognize the community and to accept it as a unit of vegetation, while its variability, although slight, is sufficient to indicate the impossibility of considering any such area of vegetation as a definitely organized unit". In a community of reasonable extent the variation of precipitation, temperature, length of day, and often of the soil, is small. The dominant plants, distributed over the whole community, exert such a uniform effect on other species that differences in the physical environment are more or less smoothed out or obscured.

Cain (29), agreeing in large part with Gleason's individualistic concept of the plant community, gave the following graphic and fundamental description: "the individual plant community has objective reality . . . sharply delimited at its borders if the habitat changes strongly in a short space. But so far as the flora of the community is concerned, it is the result of the superposition of areas of the species. Each species of the stand will have its individual and often quite different total area, and each has a more or less different ecological amplitude and modality. They live together in the particular community because they chance to have overlapping areas and are biologically successful in the particular habitat. . . . it is sometimes useful in ecology to pigeon-hole stands in approximate type groups. But it must always be remembered that community types or associations are metaphysical approximations in a field where there are unlimited variables, combinations and permutations. Between the individual stands at one end of the scale and the largest world formation types at the other (such as the broad-leaf summergreen forest), there can be few if any precise subdivisions".

The nature and distributional area of a vegetational type can not be based upon a single species, but must be based upon the entire plant population, the kinds of life-forms that are present and the structure of the community. "The limitation of each plant species requires independent investigation. The conditions which determine the location of the edge then become a composite expression of the ones found to be of greatest importance to the largest number of common plants. . . . The edge of the desert is the approximate line of demarcation between the distributional areas of two sets of plants with different life requirements". Delimitation of the desert from grassland in Arizona, New Mexico, Texas and Chihuahua is most difficult because the transition is very gradual, the most gradual of all the desert transitions (240).

In addition to gradual transitions, delimitation of communities is difficult because of irregularities in the distributional limits of species, heterogeneity of soils, lack of topographic barriers, etc.

This complexity has led in places to the lumping of different kinds of small communities into a heterogeneous complex, which tends to obscure important differences and processes. Delimitation of these small communities produces almost a fragmentation of the vegetation, but in order to secure a full understanding of the vegetation, fragmentation is preferable to lumping, as demonstrated by studies on the solodized solonetz complex in western North Dakota (121), on pattern and process in patchy vegetation (275), on fairy-ring communities (237) and others.

Delimitation of communities may be simplified by first dividing an area or region into geographic or natural areas because attention then becomes focused upon a definite and usually not too large an area. A natural area is "a geographic unit of any order of size with sufficient common characteristics of various sorts to be of some practical usefulness in biogeography" (29). The "term 'natural area' . . . is less specific than "life zone" or "community" and is used in a broad sense to denote a region having characteristics of life, climate, terrain, etc., which in a general way differentiate it from other regions. But a natural area, whether a great region like the arctic tundra or a small unit like a river floodplain, is difficult to define and not easily explained. . . . it is a complicated problem to correlate types of floras, fauna, climate, soil, physiography, and other environmental factors (107)." Several recent papers illustrate the use of natural areas in describing vegetation (264, 177, 148, 149).

ANALYTICAL CHARACTERISTICS

Analytical characteristics, in contrast to synthetic characteristics, are those features or aspects of a community which can be observed or measured directly in each stand. They include kinds and numbers of species, arrangement of species and individuals, vigor, form, numbers of individuals, height of plants, area, volume, growth rate, etc. Some of these characteristics can be measured more readily than others and are therefore classified as "quantitative", while those which are usually described are "qualitative". Synthetic characteristics denote aspects of vegetation which are based upon analytical characteristics; they are derived characteristics and depend upon data obtained in the analysis of a number of stands. They tend to summarize and generalize some of the analytical characteristics.

QUALITATIVE

KINDS OF SPECIES OCCURRING IN THE COMMUNITY (FLORISTIC COMPOSITION). A list of species of an area is known as its "flora", and the complete list of the species of a community is its first essential character (262) and a first step in its study. This involves collection and identification of plants, especially in communities new to the investigator. Often in regions where little ecological work has been done, as New Zealand (50), careful listing of species with accurate and comprehensive notes of observations may be of greater value than more detailed records which would be restricted to a few localities.

Lists of species in plant communities have considerable value for characterization and description because each species has a definite range of tolerance for different environmental conditions. Use of a single species may be valuable in delimiting and characterizing a vegetation type, such as the creosote bush (*Larrea tridentata*) in the desert in southwestern United States, or *Agropyron smithii* in some parts of the Great Plains, because these species have certain tolerance ranges for moisture, temperature, salinity, etc. When additional species are used, however, the plants become of greater value for delimitation and diagnostic purposes. For example, when *Agropyron smithii* and *Distichlis stricta* are found in one stand, and *Agropyron* and *Bouteloua gracilis* in another stand, it can be inferred that in all probability the former stand occurs on soil of greater salinity than the latter, and that these stands can best be differentiated on the basis of the occurrence of *Distichlis* and *Bouteloua*. Species of broad tolerances in western North Dakota, according to the analysis by Hanson and Whitman (122), are *Bouteloua gracilis, Agropyron smithii, Stipa comata, Carex filifolia, Koeleria cristata, Muhlenbergia cuspidata, Aster multiflorus, Artemisia frigida, Chenopodium leptophyllum, Phlox hoodii, Draba nemorosa, Gaura coccinea* and *Lactuca pulchella*. Species of narrow tolerance or of low ecological amplitude are *Stipa spartea, Andropogon furcatus, Bouteloua curtipendula, Sporobolus heterolepis, Distichlis stricta, Puccinnellia airoides, Cerastium arvense, Artemisia cana, Symphoricarpos occidentalis* and *Campanula rotundifolia*. The species in the first group occur in a number of different types, the latter in only one or very few types.

In many grassland studies only the more abundant, dominant, or what are considered the more important species have been listed or given much attention. Often a number of the less abundant species are lumped under "annual grasses", "annual forbs", "other woody species", etc., even though the plants in a group may constitute in the aggregate a considerable proportion of the vegetation. Reasons for doing this are that changes in the composition are so rapid and pronounced that accurate and complete determinations of the species are not justified, that the time spent in making such determinations can be better spent in studying the causes of the changes, and not only that the details are unimportant but that attention to them may obscure facts and principles regarding the vegetation as a unit. Undoubtedly omission of minor species may be justified and desirable in some grassland studies, but it must be kept clear that by such omissions some important facts may not be revealed and that the studies can not be considered floristically complete. It is essential scientifically, and safer practically, to include all species because some plants which appear insignificant at the time of the investigation may have value in indicating conditions which obtained in the past but do not exist at the present time, or in indicating future trends. Even though a complete list of plants may not be of any particular value for a specific investigation, the list should be published in detail so that it will be available for other studies in the same or other areas.

Cain (29) pointed out the emphatic statement of Flahault and Schröter that "all species should be considered in the characterization of the association, . . . and that no investigator has the right to prejudge the significance of certain species. The most numerous, the tallest, the most 'peculiar' may not be the most important". Shreve (240) stressed the importance of including all species in determining the nature and the distributional area of a type of vegetation. In Iowa somewhat rare species of high fidelity were the first to disappear when the prairie was disturbed, and apparently the most difficult to reestablish (4). "These exclusive plants may well be regarded as the indicators of highly developed prairie; an assemblage of the typical prairie grasses might be considered prairie, but a truly fine prairie includes these exclusive species". From 1936 to 1946 on the prairie reserve at the Iowa Lakeside Laboratory only three species, *Bouteloua hirsuta, B.*

gracilis and *Artemisia caudata,* had disappeared from the study area, the first two having been greatly damaged by the 1936 drought and replaced by annuals, such as *Festuca octoflora.* The point is that if only the dominant or the most numerous species are considered significant, effects of the environmental influences might be missed. Drew (76) made very complete lists of species as well as quantitative analysis of "domesticated" prairie (native prairie influenced by man-controlled processes) and relics of native prairie in north-central Missouri. He found that 34 of the 152 species occurring in the domesticated prairie were not in the prairie relics, and that 35 of the 153 species in the prairie relics were not in the domesticated prairie. This study emphasizes the need for including all species growing in a stand in order that the earliest effects and later changes may be noted.

In view of the large number of species reported on prairie relics by Drew, the numbers of eight to 24 species per relic prairie area in central Wisconsin, as described by Thomson (265), seem low, but the latter did not use quantitative methods. Quadrats, transects or other kinds of quantitative sampling are indispensable in securing a complete listing of all plants occurring on an area because they compel the investigator to focus his attention and thus note individuals which because of their scarcity or small size might otherwise be overlooked. Many have used definitely circumscribed study areas for listing species, estimating cover, etc., but some have failed to give complete lists (267, 78, 79, 53, 253). Canfield (34) stated that the minor species of grasses in the southwestern grasslands, of which the plants of individual species make up less than one per cent of the stand, are important because in the aggregate they represent ten per cent of the total grass cover on conservatively grazed ranges. It is significant that the more completely the range has recovered from deterioration, the easier it is to find plants of these less common species. It is therefore important to look closely for and to record these species individually because the absence of some of them denotes one of the critical points in the deterioration of the mesa range type. Another practical value of listing all species is that on a mixed grass type it appears that under good management the number of species will be greater than under poor management. On abandoned plowed lands in Colorado quadrats were found helpful in finding plants of perennial grasses among the weeds in the early stages of succession (58). Close observation is necessary during the first year after reseeding grasses in western Kansas to determine which species are present (56).

Considerable uniformity in the number of species per stand occurs in the same type of vegetation in western North Dakota (122); for example, in four stands of the *Calamovilfa* type there were 35 to 42 species, while in the same number of stands in the *Agropyron-Bouteloua-Carex* type there were 20 to 29. Xerophytic grasslands at various elevations in Colorado were effectively compared by means of species lists (210); 160 species were found in the dry grassland at 5300 ft. at the mountain front, 139 at 7500 ft. in the lower foothills, 130 at about 8400 ft. in the upper foothills, 107 around 8900 ft. in the montane zone, and 50 at 11,000 ft. in the subalpine region. On an area ⅜ of a mile square, of grazed range at 5100 ft. at the mountain front, about 50 miles north of this 5300-foot location, 112 species were found (120). In the *Andropogon scoparius* grassland on the Hempstead Plains on Long Island, N. Y., 71 species were recorded (30), while in western North Dakota, where this type is restricted usually to north-facing slopes, 50 was the average per stand (122). If the woody species had been omitted from the Hempstead Plains study, the number would have been 53. This older community was richer in species than the one on High Hill, also on Long Island (53).

The "relict method" (44) is based very largely upon the listing of species in many different areas over a long period of years. Repeated study of the composition of communities over wide areas led to conclusions such as the following: "The first intimation that the short-grass plains were not a climax in the climatic sense was obtained during the summer of 1915; this was a season of excessive rainfall coinciding with the lowest sunspot minimum for a century. The mid-grasses appeared abundantly in the cover of short-grass and in many places obscured the latter more or less completely. Since these were all perennials, it was obvious that they could not be regarded as recent invaders but must have been present in the community in less conspicuous form. This led to a search for protected areas in which they retained much of their normal importance and resulted in finding such relicts in a wide variety of situations. The protection afforded against grazing by fences usually supplied the most striking examples, as in the case of trackways, reserves and cemeteries especially, but sandy soil and rocky slopes regularly yielded mid-grass dominants".

Often in pasture experiments it is desirable for botanists, agronomists and livestock specialists to cooperate, so that different phases will be most effectively handled (297). When this is done there should be less likelihood that inadequate attention will be given to the botanical composition, as occurred in a study of pasture plants in the southeastern sandhill region in the U. S. in which chemical analyses were made of ash, protein, etc., as well as yields of digestible nutrients, but "no attempt was made to obtain definite information on the botanical composition or changes in the distribution of plants during the experiments" (92).

The need for detailed knowledge of the vegetation in order to evaluate range lands is shown clearly by Talbot (261) in a study of indicators of southwestern range conditions. Species of native plants that are insignificant on ranges which have been maintained in good condition should be closely watched because any increase in their numbers may indicate the beginning of deterioration. On poorly handled areas they may become numerous and even dominant. These species may be inferior annual grasses, such as mat grama (*Bouteloua simplex*), sixweek's dropseed (*Sporobolus microspermus*) and false buffalo grass (*Munroa squarrosa*); or they may be inferior perennial grasses, such as the three-awns, (*Aristida* spp.), burro-grass (*Scleropogon brevifolius*), fluffgrass (*Triodia pulchella*) and ring muhlenbergia (*Muhlenbergia torreyi*), and also some shrubs and forbs, as rabbitbrush (*Chrysothamnus*), groundsel (*Senecio*), Russian thistle (*Salsola pestifer*), prickly poppy (*Argemone*), burroweed (*Aplopappus fruticosus*) and pingue (*Actinea richardsoni*). In North Dakota, as shown by permanent quadrats, *Poa secunda* and *Poa canbyi* were distinctly minor in the vegetation in the western part of the State previous to the 1934 and 1936 droughts, but they increased after the droughts so that they ranked in abundance with one of the dominants, *Koeleria cristata* (299). The annual forbs also increased in abundance following the droughts. The recording of kinds of species with data on abundance, cover, etc., from permanent study areas over an indefinite period of time is essential in securing such information, and no detail about the plants is too unimportant to be omitted.

Additional examples showing the value of listing all species in a stand are given in the first paper on this subject (115).

STRATIFICATION OF ORGANISMS OR THEIR PARTS ABOVE AND BELOW THE SOIL SURFACE. Stratification is the occurrence of organisms or their parts at more or less definite levels. It is usually applied to the above-ground parts of plants, but it is also characteristic of underground parts, both roots and rhizomes. Stratification of plants is due largely to the variations exhibited by plants of different species in their life-forms and their ranges of tolerance to decreased light intensity, to soil acidity, salinity, excessive or deficient soil moisture, etc. The environments of different strata differ with respect to most factors, and this will affect the kinds and numbers of species in each stratum.

Stratification has been described in many papers on grasslands, particularly of above-ground parts, in which two or more layers frequently occur. In recent years increasing attention has been paid to the characteristic of stratification in connection with various types of problems. In irrigated pastures in Colorado, for example, orchard grass may be so abundant, due to heavy rate of seeding, that the lower layer of Kentucky bluegrass and white clover may be almost entirely suppressed (110). When grasses become more prostrate and closely packed to the ground, the denser and nearer to the surface do the root systems develop (10), so it appears that the "root habit is a reflection of the habit of the aerial parts" of grasses. Orchard grass is especially subject in Great Britain to this "pot-bound" or root-bound condition. Unquestionably grazing has considerable influence upon the stratification of plants. Grazing and mowing, by removing the taller growing species, give opportunity for plants in the lower strata to increase in abundance. In ungrazed prairie relics in Missouri *Antennaria neglecta* and *Viola sagittata* comprised 1.4% and 0.4%, respectively, while in the grazed or mowed prairie they made up 9.3% and 3.1% of the abundance (76). Other species also more abundant in the latter were *Trifolium pratense, T. repens* and *Oxalis stricta,* mostly components of the lowermost stratum. In Iowa and other parts of the prairie *Poa pratensis* is often the chief constituent of the lowest layer (4).

Bromus tectorum has received much attention in recent years because of its increased abundance over wide areas of the grasslands in the western States. It also forms an understory with other grasses and forbs in association with *Artemisia tridentata.* The sagebrush exerts a suppressing influence on all of the plants in the stand when its crowns cover 40% of the ground (222).

Increasing research is being given to root systems and their stratification since the basic studies of Weaver (278, 279). In much of his work the trench-pick method was used (276, 278–281). Pavlychenko (192, 193) excavated large blocks of soil and then painstakingly washed away the soil from the roots, and others have added refinements (259, 263) or modifications (242, 101).

The earlier work on root systems was concerned largely with descriptions of the root systems, including layering, of various species, while in recent work layering is used more or less as a tool to explain vegetational processes and as an aid in solving problems. In spite of its shallow root system, *Bromus tectorum* has been able to invade and compete successfully with shrubs and deep-rooted perennial grasses. In Utah, for example, its roots penetrate to only about six inches, while those of the perennials *Agropyron inerme* and *Helianthella uniflora* reach four and five feet, respectively (123).

Grazing and mowing, especially when excessive, have pronounced effects upon root systems. In an overgrazed low prairie near Lincoln, Nebr., the roots of *Buchloë dactyloides* were concentrated for the most part in the upper two feet, although a few penetrated to nearly seven feet, while those of the intermixed *Agropyron smithii* were distributed more uniformly to a depth of about eight feet. Under protection from grazing *Agropyron* replaced *Buchloë* almost entirely, due at least in part to the advantage of having the deeper roots during dry periods (49). On heavily grazed range in Utah the average root depth of *Agropyron inerme* was 44. 2 cm., on protected range 65.2 cm. (255).

Layering of root systems may be caused by variations in the salt concentration at different depths in the soil. In the vicinity of Milford, Utah (251), roots with large branches of *Bouteloua gracilis, Hilaria jamesii, Oryzopsis hymenioides* and *Sporobolus cryptandrus* were located entirely in the upper 30 to 45 cm. where the salt content was less than 1000 p.p.m.; some of the larger roots of *Atriplex confertifolia, Chrysothamnus stenophyllus* and *Eurotia lanata* extended to depths of 40 to about 65 cm. where the salt content was over 1000 p.p.m.; while roots of *Artemisia* *tridentata* and *Grayia spinosa* reached 80 to 90 cm. where the soils were more porous and had less salt.

The roots of six species of pasture and turf grasses in New Jersey declined in abundance very rapidly from the first to the sixth inch (242). Application of fertilizer on the surface, also in New Jersey, caused an increase in the roots near the surface, and a decrease below two inches. This concentration of roots near the surface was considered important in erosion control (125). In a silt-loam soil underlaid by sandstone at a depth of 16 to 18 inches at Morgantown, West Virginia, the weights of roots of five species of grasses were greatest in the upper three inches, decreased rapidly in the next three inches, and progressively below that. In the upper three inches the roots of deer's tongue (*Panicum clandestinum*) exceeded in weight the combined weight of the other four species, namely *Bromus inermis, Dactylis glomerata, Poa pratensis* and *Phleum pratense*. At depths, however, of nine to 12 inches and at 12 to 18 inches the roots of *Bromus* weighed most, followed by *Dactylis* and then *Panicum*. Deer's tongue, sometimes considered a weed, may be of considerable importance in erosion control because of this concentration of roots near the surface and in the reclamation of acid-eroded soils which are not capable of supporting satisfactory stands of other species. Deer's tongue has been found to tolerate a pH value as low as 3.5 in the soil surrounding its living roots (101).

The influence of grassland upon the physical and chemical properties of soils is receiving more quantitative study than a decade ago, especially in relation to improvement of soil fertility and control of erosion. In a silty clay loam soil near Lincoln, Nebraska, blue grama grass, buffalo grass, brome grass, switch grass, Kentucky bluegrass and orchard grass were found more effective in a seven-year period of maintaining a high content of organic matter than side-oats grama, big bluestem, a weedy cover of mostly annual weeds or a cultivated field (166, 167). Considerable difference was noted among species in their effectiveness in stabilizing soil aggregates during this period and in the persistence of the aggregates during a two-year period of cropping to small grain after plowing up the seven-year-old sods. In the zero to three-inch layer of soil the buffalo grass plot showed a markedly smaller decrease in the percentage of water-stable aggregates greater than

0.25 mm. than that in the plots of other species. The decline was from 34.1% to 22.5% during the two years of cultivation. The initial content of the aggregates was not so high in the buffalo grass soil as in that of other plots, but after the two years it was the highest. The reductions in other plots were from 52.4% to 23% in side-oats grama, 40.1 to 20.5 in switch grass, 43.9 to 21.5 in orchard grass, 43.2 to 20 in big bluestem, 45.3 to 19 in brome grass, 45.7 to 18.5 in Kentucky bluegrass, 48.6 to 18 in blue grama, 38 to 2 in the weedy plot. The ability of grasses to stabilize soil aggregates is evidently related to the type of root development of different species, and the persistence of the aggregates to the chemical effects of their decomposition products. Much additional work is needed to learn about the specific effects of various species and about application of the data to soil improvement.

PERIODICITY (PHENOLOGY, ASPECTATION). By "periodicity" is meant the occurrence at various seasons of the year of plant processes, such as photosynthesis, growth, pollination, blooming and ripening of fruits and seeds, and the manifestations of these processes as leaf formation, shoot elongation, appearance of flowers and dissemination of seeds. These processes and manifestations appear regularly as a rule during definite periods of the year and are essential features of the life cycle of individuals, so there is a certain periodicity in their occurrence. This periodicity is due to the inherent genetic and physiologic characteristics of the plants under the influence of the environmental factors in which the plants are growing. The term "periodicity" refers more to the fundamental recurrence of these processes and their manifestations, while "phenology" refers more to the appearance of the manifestations at certain seasons of the year. The aspectation of a community is due to the inherent characteristics of the plants of the individual species, and the resulting appearance of the community is due to the manifestations of many individuals in one or more species, acting as individuals, growing together in a stand, and is not due to any response of the community as a whole.

Greater precision marks the use of this characteristic in recent years. In determining the time when a range type is ready to be grazed ("range readiness"), much use has been made of certain stages of growth of the most important forage plants (231, 258). In a detailed study (60) of plant development in relation to grazing in the Wasatch Mountains, Utah, dates of the following stages were obtained: grasses—snow disappearance, herbage height at six inches, flower stalks evident, heads beginning to show, flowers in bloom, seeds ripe, seeds disseminated; forbs—disappearance of snow, flower buds evident, full bloom, seeds ripe, seeds disseminated, plants dried up; browse species—disappearance of snow, flower buds bursting, leaf buds bursting, full leaf, full bloom, fruits ripe, all leaves and fruits dropped. It was noted that each species has its specific rate of growth and development, and that each developmental stage of each species has its own rate of development. The earlier growth stages developed more rapidly than the later ones, and the rate of development was delayed ten to 14 days for each increase of 1,000 feet in elevation. Although growth is more rapid after it starts at the higher elevations, a longer period is needed to reach the seeds-ripe stage. Inception of active growth of grasses in the spring varied as much as 45 days, and the date when the key grasses reached a height of six to eight inches (range readiness) varied up to 47 days in different years. In this study a method was also worked out for forecasting the dates of various stages from the date of snow melting.

Bluebunch wheatgrass in southern Idaho over a period of ten years varied in the date of inception of growth in the spring from March 20 to April 24, in herbage height of six inches from April 26 to June 1, in seeds formed May 12 to July 10 (61). In some species, such as mountain brome, slender wheatgrass, sticky geranium and niggerhead in the Wasatch Mountains in Utah, herbage growth begins early in the spring under the snow, and root growth immediately after the winter snow disappears (163). Secondary herbage growth occurred in the autumn when the foliage formed in the spring was drying. Shoot buds formed in the crown at this time live through the winter under the snow and produce the early spring growth. Storage of carbohydrates was at a minimum during the periods of greatest growth, and at a maximum at completion of the secondary herbage growth. Growth early in the spring was dependent upon the carbohydrates stored in the autumn.

A community is given a definite aspect at different seasons of the year by the vegetative growth, the blooming especially, and the fruiting of various species. Numerous descriptions of vegetation include the course of seasonal development under the four chief aspects: prevernal, vernal, aestival and hiemal (hibernal) (120, 289). The effect of burning mixed prairie vegetation in central Oklahoma was to delay the appearance of the prevernal aspect and to prolong the vernal, but there was little difference in the later aspects, as compared with the unburned vegetation (37). A number of forbs occurring in Illinois, Texas, Oklahoma, Nebraska, South Dakota, Minnesota and Manitoba have been classified into the four aspects (38). The changes occurring monthly in the Western Cross Timbers of north-central Texas have been described and the value of such phenological data stated thus: "Satisfactory results from pasture mowing, deferred grazing, rotation grazing, prescribed burning, reseeding, seed harvesting, diagnosis of causes for livestock gains and losses in weight, or deaths from plant poisoning all depend upon accurate knowledge of the stage of growth and composition of the vegetation on a range at a certain time" (79).

In a recent analysis of the grasslands of southern British Columbia (267), a few characters including dates of first growth, blooming period and ripe seed stage, of some of the principal species were used. There were three phenological groups of species. The first group, in which were *Agropyron spicatum* and *Stipa comata,* started growth early in the spring, developed fairly rapidly and completed vegetative growth and often reproductive development before summer drought became severe. During the dry period they became more or less dormant, and growth was renewed in the autumn if the rains were adequate. The second group, which included low perennial and annual species, *e.g., Poa secunda, Bromus tectorum* and *Antennaria dimorpha,* also started growth early, grew rapidly and were fully cured before arrival of the drought period, renewing growth in the autumn if moisture was ample. The third group, comprising desert shrubs, *e.g., Artemisia tridentata* and *Chrysothamnus nauseosus,* began growth a little later than the species in the first two groups and continued to grow throughout the entire frost-free season. These differences were related to their root development. The root system of *Agropyron spicatum* is extensive and penetrates to about four feet, the roots of the second group of plants rarely penetrate below one foot, and those of the shrubs penetrate deeply where moisture is available throughout the growing season (276).

Early spring growth gives some species a great advantage in the competition for limited supplies of soil moisture, as noted in *Poa* spp., *Agropyron* spp. and *Bromus tectorum,* especially as compared with *Bouteloua gracilis.* Often the early and rapid growth of the misnamed "nurse" crops, such as winter rye or other cereals, exerts a pronounced suppressing effect on later and slower growing perennial pasture plants. *Poa pratensis* and *Agropyron smithii,* for example, had a great advantage in their early growth and maturation in competing with *Andropogon* spp. and other prairie species (68).

The term "periodically saturated" is expressive in connection with the seasonal dominance of different species in the various aspects (18). This term also indicates the difficulty of invasion by new species into a community because it is already fully occupied, and many species already present in the community could not survive unless their blooming and other activities came at different seasons. Detailed, continuous and systematic records of periodicity over a period of years are essential for a full understanding of a community; occasional, irregular observations are by no means sufficient.

VITALITY (VIGOR OF INDIVIDUALS OF A SPECIES, GEDEIHEN). "Vitality" refers to the condition, or to the degree of vigor, of a plant. Acquaintance with the appearance of normal plants at various stages of growth is necessary in order to be able to appraise the degree of vigor. The presence of thrifty individuals may indicate that they are invading the stand, or it may mean that the conditions of the habitat are especially favorable, so they may become dominants. Species represented by weak plants, which may fail to reproduce, may be losing out in competition with others and are in the process of being replaced. If it is desirable to maintain the species in the community which is being mowed or grazed, some change of treatment may be called for. Recognition of the first signs of reduced vitality is most important in the management of grasslands because something can often be done to change the trend before it is too late. Species which are represented by few individuals in a stand merit special attention because they may indicate the end of a previous stage of succession, the early arrivals of a new stage, of changing conditions such as decreased or increased soil moisture, some kind of harmful grazing practise, etc.

A number of criteria may be used in determining the vitality of plants, *e.g.*, *a*) rate of growth renewal in the spring, or following grazing or mowing; *b*) rate and total amount of growth during the season, especially in height; *c*) quantity of foliage produced, including length and width of leaves, or area; *d*) color of leaves and stems; *e*) turgidity of leaves and stems; *f*) degree of damage by bacteria, fungi or insects; *g*) appearance of flower stalks at the usual season, and their number; *h*) extent of the root system, length and diameter of individual roots; *i*) area of crowns or herbage of tussock grasses and turf-formers; *j*) boundaries of bunches, mats or patches, sharply defined boundaries usually indicating thriftiness; irregular ones may indicate declining vigor; *k*) number of new stems, offshoots, rhizomes, etc., which are appearing; *l*) increasing number of stems and leaves per unit area (density), probably denoting increasing vigor; *m*) extent of dead portions of bunches or mats; *n*) age of plants in the stand, vigor being good if young and old plants are both present; but seedlings may be absent due to competition; *o*) number and condition of flower stalks, flowers or fruits (287, 219). The most obvious sign of improvement of a meadow in poor condition in the mountains in eastern Oregon and Washington was the establishment of young thrifty perennials among the annual forbs and grasses (219). Improvement was also indicated by the increase in vigor of perennial forbs already in the stand, such as agoseris, dandelion, western yarrow, groundsel, cinquefoil and little-flower pentstemon. Some of the scattered newly established plants were mountain brome, oval-head sedge, prairie junegrass, Kentucky bluegrass, Nebraska sedge and Baltic rush.

A common classification of the degree of vitality of species in the community is: *a*) species represented by well developed plants which regularly complete their life cycle; *b*) species with vigorous plants which increase in number but do not usually complete their life cycle; *c*) species with feeble plants but spreading, although never completing their life cycle; *d*) species represented by plants which occasionally germinate but are not increasing, as many ephemeral plants (18). In an *Andropogon scoparius* stand on Long Island 20 species were classified in class *a*, three species in class *b*, one species in class *c*, two species in class *d*, and two species not classified. The large number of species in class *a* was interpreted to indicate that the dominance of *Andropogon* was not so great as to prevent the flowering and seed-production of many other species. This example suggests that greater use could be made of this or a similar classification in analyzing communities (53).

A number of grasses form more roots in proportion to tops at lower than at higher temperatures. This may be important in survival during dry periods, since the plants with the greater root systems would have the highest degree of vigor. It is well known that grasses with large amounts of stored carbohydrates recover more rapidly after severe and frequent grazing, or close clipping, than those low in carbohydrates. Sugars and fructosan are common forms of carbohydrates which are stored in the roots and in the lower parts of the stems (94). Relatively high concentrations of sugars are required in the basal organs and newly developed shoots of mountain bromegrass in order that the vigor will be sufficient to enable the plants to survive the winters in Utah mountains (162). This is directly related to grazing practises; as maximum storage occurred in the autumn after the herbage growth had been completed and temperatures were declining, if the plants had been too closely grazed the vigor of the plants would be reduced. *Agropyron spicatum,* which has been replaced to a great extent in northern Utah by *Bromus tectorum* and *Artemisia tridentata,* is most subject physiologically to damage by overgrazing during the month of May (256).

Numerous experiments have been conducted which show that the maximum vigor in seedlings of grasses and legumes is secured when the seed is planted at 0.25 to 0.75 inch deep, depending upon moisture conditions in the surface soil (257, 248, 161, 204, 160, 178, 84). Many of the weaker seedlings die as a result of competition with more vigorous plants, but under favorable conditions the lower degree of vigor may be overcome in a few months. The larger plants may be first beneficial and then harmful to the vigor of the seedlings, as in the prairie near Lincoln, Nebraska: "Seedlings present little foliage until new shoots of established vegetation are sufficiently enlarged to reduce greatly the light near the ground level. As a result they are unable to form adequate leaf surface for photosynthesis. Their roots are starved for carbohydrates and deprived of water by the more efficient absorbing systems of mature, rapidly transpiring plants. Incapable of growing up into the light or of extending into the subsoil for water, seedlings usually lead a suppressed existence and perish in the first drought of summer". Vigorous forbs in the second or third leaf stage showed a survival of 80 to 100% during the following winter (16).

Many experiments have been conducted on the effect of the closeness and frequency of clipping or mowing upon the vigor of grassland species (150, 285, 290, 258). In general, the closer and the more frequently the clipping is done, the more do the plants lose in vigor. One example of recent work is given (127). In eastern Montana under a frequency of two weeks and a clipping height of three cm. above the surface, continued for four years, the height of vegetative stalks of *Agropyron smithii* was 24.9 cm., the number of stems per plot of 314 sq. cm. was 37, and the weight 5.5 g.; as compared to height of 26.7 cm., 114 stems, and 16 g. when cut at the same height every four weeks. When clipped at eight cm. every two weeks the height was 30.0 cm., 123 stems, yield 20.0 g. The control clipped at three cm. at the end of each season, was 32.1 cm. tall, 189 stems, and 33.0 g. These results were similar to those obtained in North Dakota (233) and in Wyoming (150). All of the treatments were too severe for maintenance of the plants. The vigor of *Bouteloua gracilis* plants was also reduced after four years of clipping, as shown by the following measurements in the fifth year after clipping every two weeks at two cm.: height of seed stalks, 23.7 cm. (24.4, 26.6, 30.3); height of basal leaves, 11.2 cm. (13.1, 15.4, 15.9); weight in grams per 100 sq. cm., 8.5 (10.5, 16.6, 27.1). The figures in parenthesis are, respectively, for clipping at two cm. every four weeks, clipping at four cm. every two weeks, and the control, clipped at two cm. at the end of the season.

The ecological amplitude of a species, or its tolerance (106: 332), the capacity to grow and reproduce within a certain range of environmental conditions, is a factor in vitality because as the limit of tolerance of a certain condition is being approached for a certain species, the individuals of that species will probably have reduced vitality, especially in comparison with individuals of species which are not near their limits of tolerance. Some species show greater tolerance of close mowing and grazing than other species. *Andropogon scoparius* suffers more under drought than *Agropyron smithii* and *Bouteloua gracilis.* In the alkali part of an irrigated pasture the plants of Kentucky bluegrass would be in very poor condition, if they were able to survive at all, while those of *Agropyron smithii* would be thrifty due to the greater tolerance of the latter for alkali; in the non-alkali parts the Kentucky bluegrass might be more thrifty than the wheatgrass. A few species, e.g., *Redfieldia flexuosa,* are able to tolerate burial by one to several feet of inblowing sand.

The vigor of grasses may be measured (287) by transplanting blocks of sod four to eight inches thick, before growth begins in the spring, into a greenhouse and observing their growth for four to six weeks. The soil is then washed away, the growth of both tops and roots measured and weighed. In one test of eight species of grass the dry weight of tops of weak plants was 32 to 84% less, and the roots 28 to 94% less than the respective weights of plants in fair to good vigor. In northern New Mexico (91) blue grama grass in full vigor had roots extending to a working depth of 1.5 feet and maximum depth of 3.5 feet, while the plants in low vigor had a working depth of only six inches and maximum depth of 1.5 feet. The maximum depth of greatly weakened plants was only 0.5 to 0.75 foot, and the working depth one to two inches.

Recent research is showing the relations between the genetic composition of species and strains and vitality. Five climatic ecotypes, for example, of *Deschampsia caespitosa* subspecies *genuina,* all of which were morphologically similar and could be differentiated only physiologically by experiment, were distinguished on the basis of their reactions in vigor, including height of the tallest stems, number of flower stalks, diameter of the clump area, flower bearing, fruiting, earliness, survival, susceptibility to frost and to disease (152). The ecotypes, rather than the species, are the true indicators of the ecological conditions to which they are fitted. Usually it can be expected that an ecotype growing outside its natural environment will not be so vigorous as one within it; the southern strains of brome grass, for example, have been demonstrated to be more thrifty in Nebraska and Kansas than northern strains. Under a 13-hour photoperiod a strain of side-oats grama from near San Antonio, Texas, flowered rapidly and persistently, and the vegetative growth was rapid, while a strain derived from Cannonball, North Dakota, flowered weakly, and the growth was very limited (187). This does not mean that the ecotype is restricted to the particular area in which it is native, because distant areas may have closely similar environmental conditions. Various parts of the Ukraine steppe are climatically analogous to sections of Nebraska and South Dakota, and other climatic analogues have been worked out (186, 116). Agricultural expansion in many areas will benefit by the introduction of plants from areas in countries having similar environmental conditions ("homoclimes"), as, for example, plant introductions for northern semi-tropical portions of Australia from South America (130).

In many cases deviations in the number of chromosomes adversely affect the vigor, appearance and fertility of the plants, as in orchard grass. In some strains of perennial ryegrass, meadow fescue, white clover, red clover and alsike clover the plants with double the number of chromosomes differed from plants with the normal number in physiological reactions, morphological characters and chemical composition. None was superior to the normal types (94).

LIFE-FORMS INCLUDING VEGETATION-FORMS, GROWTH-FORMS, HABITAT-FORMS. The particular life-form of a species is due primarily to heredity, and it represents the adjustment of the vegetative portions of the plant body and its life history to the environmental conditions. At times pronounced changes in conditions may cause a change in the life-form, as, for example, when *Bromus catharticus,* a four- to five-year perennial in South America, became an annual when introduced into the United States (292). Usually a community, except the simplest, contains several kinds of life-forms because different kinds are equally or sufficiently well adapted to live within the conditions of the habitat, and, furthermore, most communities offer a variety of local and microhabitats. For example, *Agropyron smithii,* a rhizome-grass with erect shoots six to twelve or more inches tall, grows in the same community in the Great Plains as *Stipa comata,* a taller perennial bunchgrass; *Bouteloua gracilis,* a rhizomatous matformer; *Festuca octoflora,* a small annual; and a variety of annual and perennial forbs.

The term "synusia" has been used to designate a community of species which belong to the same life-form group, as a tall shrub layer in a forest, or an aggregation of crustose lichens (18, 27). The term "union" refers to an elementary unit of homogeneous vegetation in which most of the species belong to the same life-form and usually occur in a single layer. Two unions may occur in the same layer, as in the Russian steppe where one union consists of perennial tufted grasses, such as *Stipa* spp., *Festuca sulcata* and *Koeleria cristata,* and another union consists of therophytes, such as *Eragrostis poaeides* and *Setaria viridis* (156).

Raunkiaer's biological spectrum is valuable in expressing differences and similarities among communities. Usually grasslands are rich in hemicryptophytes, as, for example, in an Agrostidetum in So. Cévennes where 81% of the species were in this class and in a Trisetetum where there were 89% (18), but only 8% and 2%, respectively, of therophytes. A grassland community near Bombay, India, dominated by *Themeda triandra* and *Pseudenthistiria heteroclita,* differed greatly from these, as most of the 84 species present were therophytes (13). Instead of using species lists as a basis for biological spectra, the sum of the frequencies of various species representing a certain life-form may be used (23). In a total of 100 quadrats in ten stands in hardwood forests in Minnesota, *Carex pennsylvanica* occurred in 94, and since it is a cryptophyte it contributed 94 frequency points; while *Clintonia borealis,* occurring in only three quadrats contributed only three points to the total frequency of cryptophytes. According to this method it was concluded that the Minnesota forests show the influence of the drier more continental climate, as compared with the mixed mesophytic forests of the southern Appalachians.

Iverson (133) proposed an interesting classification of habitat-forms which was based on a large amount of field work in Denmark and Greenland, especially in grasslands, and upon ideas contained in other systems, particularly Raunkiaer's, as well as upon developments in plant physiology (170, 171). Iverson stated that Russian investigators have used similar systems (138, 212). Iverson's system is useful in showing differences between and in classifying grasslands. It was based largely on the relationship of plants to water. "Each plant species possesses an 'ecological individuality' (212) and can therefore serve as an indicator of definite ecological conditions. *Juncus-gerardi*-rich meadows furnish an example. In strongly saline places the companion species of *Juncus gerardi* are well developed halophytes as *Salicornia*. Where the salt content is weaker *Agrostis stolonifera* and other species appear. Where the salt content is very low a mixture of *Triglochin palustris, Heleocharis uniglumis, Trifolium fragiferum* and *Carex glauca* is present. These accompanying plants are so-called botanical indicators of the amount of salt concentration; at the same time, for example, *Triglochin palustris* indicates much moisture, but *Carex glauca* little moisture. If the indicator value of the various meadow plants is known for these meadows then one has a means to delimit and to classify the different meadow types on an ecological basis".

Iverson's classification has four main divisions: land plants, swamp plants, amphibious plants, water plants. Land plants are divided into five classes: (*A*) seasonal xerophytes, as *Sedum*, which have shallow root systems and tolerance to endure long periods without absorbing water; (*B*) euxerophytes, with well developed root systems similar to the tops and quickly suffering wilting when water absorption ceases, comprising four sub-classes: (*a*) succulents, as *Eryngium maritimum;* (*b*) scleromorphs, as *Psamma arenaria;* (*c*) plants with leaves and stems covered with a felt-like layer of hair, as *Artemisia* spp., and (*d*) mesomorphs which are transitional between the euxerophytes and the mesophytes, as *Lathyrus maritimus;* (*C*) hemixerophytes, with a weaker root system and quickly wilting when water absorption ceases; similar subdivisions can be made as in the euxerophytes; (*D*) mesophytes, with weak root systems, wilting when water absorption ceases, and mesophytic shoots, wilting partly with loss of turgor, as *Avena elatior;* (*E*) hygrophytes, similar to class four but wilting completely on loss of turgor, as *Caltha palustris.* Swamp plants, characterized by well developed aeration tissue, are also subdivided into four classes by Iverson.

This classification was used to good advantage by Iverson in analyzing many grassland communities, of which the following is one example. A *Deschampsia caespitosa* wet meadow showed the following spectrum of habitat forms (percentage of species in each class): euxerophytes, 0%; hemixerophytes, 33%; mesophytes, 48%; hygrophytes, 19%. An *Agrostis tenuis* dry meadow contained: euxerophytes, 12%; hemixerophytes, 15%; mesophytes, 62%; and hygrophytes, 8%.

"Growth-form" is a desirable term to employ in respect to the different degrees of development of plants in the same species due to variations in environmental factors. In abandoned fields in Colorado Russian thistle may be found ranging in height from an inch or two to plants a foot or more tall, while in adjacent grasslands the tallest plants are only two or three inches. Individuals of any species of grass vary in height, spread, number of stems, etc., in every stand, so it is well to have a term to use in connection with the degree of growth attained under different conditions during the same season. Studies of ten species of grasses and forbs for four years, and of 13 additional species for one year on high summer range in Utah (41) "prove clearly that these species under such conditions do not have a growth form that even approaches constancy. Growth form for the same species was found to vary markedly between samples from different years, from different elevational zones, and from different sites. Each species exhibits its own characteristic growth response and is influenced by exposure, soil, shading, moisture conditions, temperature, and other factors of the environment". Wide variation is expected in the total forage produced and in the proportion which is consumed when the range is grazed to a given height, and large errors can consequently be expected to result from the use of tables which give the average percentage of forage volume above given heights of the plant in connection with the volume-height method of determining range utilization.

The "use groups", recently devised (108) for testing grasses, is an application of the life-form characteristic. In this method species or strains are compared within certain categories; for example, grasses that show promise for spring-fall use are compared with other grasses in this same group and not with those intended for summer use. Each use group has a standard to which others are compared; *Agropyron trachycaulon* is the standard for *Bromus marginatus, Elymus canadensis, E. glaucus, Agropyron subsecundum* and *Lolium multiflorum* because of similar characteristics, such as longevity, high seeding capacity, rapid germination and vigorous seedlings. "It would be disastrous to compare any of them with grasses in the groups exemplified respectively by" *Festuca idahoensis, Dactylis glomerata, Stipa pulchra* or *Agropyron smithii.*

A number of studies describe how growth-forms are affected by environmental conditions, such as soil moisture (110, 282); length of day, the prostrate habit of growth of *Poa pratensis* being a short-day effect (201); salinity; and application of fertilizers. Leys in Britain may not change much in botanical composition as they become older, but a great change may occur in the life-form (10). As a mat of roots and rhizomes accumulates beneath the turf, the tillers increase greatly in number, and the individual tillers become smaller. The grasses meanwhile become smaller, more prostrate, and form a short dense turf. This is accompanied by denser and shallower growth of the root system, and thus an intensely over-crowded condition results, with decline in the yield of forage.

Considerable attention has been given in recent years to the selection of strains of grasses and legumes which have life-forms that are of greatest value for grazing, for hay, for special situations, or for general use. One of the important factors in achieving an increase of 62% in the output of starch equivalent per acre from some British grasslands during the last world war was the use of leafy recumbent strains (142). In eastern Canada, where the pastures consist of a mixture largely of timothy, red top and bluegrass, with or without wild white clover, the protein and fiber contents failed as indices of nutritive value. The leaf-stem ratio appeared to be more important as a criterion because the digestibility of the herbage for steers and sheep followed this ratio quite closely. A wide ratio indicated high digestibility which decreased as the proportion of stems in the herbage increased (63). On the range it is generally recognized that sheep prefer to graze leaves rather than stems (55), which indicates that the leafy life-form strains are preferable from the nutritional viewpoint. The Fairway strain of crested wheatgrass is smaller, more decumbent, has finer leaves and stems, and is lower in lignin than the standard strain at some stages of growth. In Montana steers gained more on the Fairway than on the standard strain (239). The Parkland strain of smooth bromegrass has a tendency to grow in bunches and is less apt to become sod-bound than the regular strain. Biennial sweet clover is often preferred to the white because of its lower more prostrate life-form which enables some of the flowers to survive grazing and thus produce seeds.

Taller deeper rooted life-forms of grasses and legumes are preferable at times because the total yields of dry matter and protein are greater than of low forms (185), and they also permit greater versatility for pasture, hay or silage, such as orchard grass, alfalfa and Ladino clover, as compared with low forms, Kentucky blue grass and white clover (94, 98). Due to its habit of growth more leaves and stems of alfalfa are removed in grazing than of Ladino clover. Therefore grazing of alfalfa should be managed so as to allow sufficient time for growth and re-storage of foods. Forage yields of selections of an erect type and low-crown pasture type of alfalfa were approximately equal, but S_1 progenies of the low-crown clones yielded more than those of the erect type (302). Late flowering forms have been recommended because of their greater value for grazing or mowing, such as the S-48 selection of timothy (245), or for their greater protein content, as in Kentucky bluegrass (94).

Relationships have been found between the life-form of grasses and resistance to fungi. In northwestern United States several species of forage grasses are commonly affected with head smut (*Ustilago marginatus*), and two strains of *Bromus marginatus*, which can be distinguished by their growth habits, have been found outstanding in resistance (147). One type is early maturing and fairly leafy, the other is late maturing and very leafy. The latter is preferred for mixtures with sweet clover. In England red and white clovers are more favorable for the development of sheep worms than grasses because the grasses present more obstacles, especially the lower leaves, to the ascent of the animals.

SOCIABILITY (GREGARIOUSNESS) AND ASSOCIATION OF SPECIES. "Sociability" or "gregariousness" refers to the space relationship of plants, or how closely together the individual plants or shoots grow. Species that spread only by seed often show a high degree of sociability, especially in early stages of succession, as in abandoned fields where annual weeds become very dense. The capacity of these plants to form dense groups is related to the number of seeds produced, the mobility of the fruits or seeds, survival and germination of the seeds, survival of the seedlings, and the ability of the seedlings and mature plants to survive intense competition. *Bromus tectorum* owes much of its success in invading western grasslands to its sociability. Propagation by rhizomes and runners favors gregariousness and often gives species possessing them an advantage in invading new territory. The new shoots near the tips of the offshoots are sustained by the parent plant while new roots and leaves are developing, and thus are better able to survive the competition of plants already present or to reach the moister soil below a dry surface layer. *Agropyron smithii* furnishes an excellent example of this in its invasion of weed-covered fields and grasslands greatly damaged by overgrazing, drought or deposition by wind-blown soil (68).

The degree of sociability that a species will develop in an area is determined largely by the prevailing environmental conditions and the biological equipment, including the ecological amplitude, of the species. In eastern North Dakota soil moisture is usually ample for *Agropyron repens* and *Poa pratensis* to form patches and suppress *Agropyron smithii,* but in the western part of the State, where the moisture is much less, these two species do not grow vigorously nor densely enough to hold their own with *A. smithii.* In newly seeded irrigated pastures in Colorado the rapidly growing, larger seedlings of orchard grass are able to produce dense stands very quickly and suppress the slower-growing, smaller seedlings of *Poa pratensis* and *Bromus inermis* (110). Ryegrass and redtop have similar effects on Kentucky bluegrass and Chewing's fescue in lawns and turfs (83), and many similar instances have been noted (269). The use of "nurse crops", such as rye, in seeding pastures usually results in a dense stand of the cereal which suppresses the slower-growing and often shade-intolerant grass seedlings (110, 113, 293, 257, 225, 161). Topographic and soil conditions cause variations in sociability of the same species in different sites (122). The degree of sociability was higher under deferred and rotation grazing than under continuous grazing in Colorado for *Agropyron smithii, Schedonnardus paniculatus, Eurotia lanata* and *Senecio perplexus;* but the reverse was true for *Bouteloua gracilis, Aristida longiseta, Artemisia frigida* and *Astragalus missouriensis* (120). *Agropyron spicatum* grows in a high degree of sociability in the three grassland zones ranging from 1400 to 2800 feet in British Columbia (267), and it was shown to be denser on protected range than on heavily grazed range in Utah (123).

Much study has been given in recent years to the statistical analysis of plant and animal distribution, i.e., to the question whether they are dispersed at random (Poisson distribution), or whether they show over-dispersion (hyperdispersion, contagious distribution) or under-dispersion (hypo-dispersion, negative contagious dispersion). Random distribution indicates that the arrangement of plants on an area is of such a pattern as would result from chance or on the theory of probability, and was called "Poisson distribution" after its discoverer. For example, if an area were divided into 100 plots, the first seed of a plant would arrive and germinate on any one of the plots entirely by chance, regardless of previous occupants. If one-fifth of the plots already had plants of this species on them, the probability is that one of the next five seeds would fall on the plots already occupied and the other four would fall on plots not occupied by this species. Over-dispersion (64, 40, 15) occurs when the individuals of a species occur in groups so that some units of an area would be densely crowded, while other units would be sparsely populated. Under-dispersion refers to a distribution which is more regular than chance occurrence, as a planting of grass plants in rows on dunes, or a corn field. Evidently individuals of different species may occur in any one of these kinds of dispersion in a specific community, and the same species may be found in any of these kinds in different communities. The local distribution of *Ribes* was found to be intermediate between the Poisson and over-dispersion types, but closer to the former (93). This was due to the dispersal by chance of the original seeds by birds, followed by aggregation of individuals around the original invaders.

One of the characteristics of true Poisson distribution is that the variance (the square of the standard deviation) is exactly equal to the mean (93). When organisms diverge from this, over-dispersion results, not only in an unexpectedly large number of unoccupied quadrats but also in the presence of a larger number of heavily populated quadrats than would be expected under random distribution, and the variance becomes greater than the mean. Clapham (40) suggested that the measurement of divergence of a given distribution from randomness be based on the relation between the observed variance and the actual mean. He called this measure the "relative variance" (variance divided by the mean), and he listed and charted values for 44 species in

Steiger's (250) analysis of a prairie near Lincoln, Nebraska. Eight species were found to have relative variances below two, 18 species varied between two and ten, and in 18 other species the variance was more than ten times the mean.

Cole (51) pointed out that not only plants (which increase either by seed or vegetatively) but also animals that produce litters or egg masses tend to become concentrated near the parent. Cole prefers the term "contagious distribution", following Pòlya (206), to "empirical distributions" of organisms, whatever the cause of the departures from randomness, the ecological implication being that the presence of one or more organisms within a sample unit influences the probability of other organisms occurring in the same sample. He also prefers "negative contagious dispersion" to "under-dispersion". Cole advanced the hypothesis that over-dispersion is made up of the intermingled random distributions of groups containing various numbers of individuals, such as pairs of birds, litters of foxes, etc., and this would also apply to aggregations of individuals around the first weed invaders in a field, as illustrated by leafy spurge or field bindweed. The primary grouping has been recognized often, but study of the distribution has been limited to individuals. Usually there is a pronounced tendency for individuals of a species to remain together, so the unit of distribution may well be considered something more than the single individual in both plants and animals. If these primary groups could be recognized, perhaps by correct sampling procedures, it might become evident that these groups are actually randomly distributed and that many cases of apparent contagiousness are really the result of unsuitable sampling.

Ashby (8), however, concluded that a most noteworthy advance in statistical ecology of the 11 years preceding 1948 is the knowledge that individuals are not as a rule normally or randomly dispersed and that over-dispersion is the rule much more often than under-dispersion. He agrees in large measure with Cole (51) and Fracker and Brischle (93) that, according to the little available evidence, it appears that a species which has no "biological predisposition" toward over-dispersion is randomly distributed when it first occupies an area. As it increases in density it loses its randomness and becomes over-dispersed, and its distribution may become random again during its final stages if it tends to disappear from a community owing to competition or to the course of succession. So there may be a "cycle of dispersion" accompanying colonization, establishment and displacement of a species; but this requires confirmation. Ashby also stated that over-dispersion of a species at high densities, when not caused by soil heterogeneity or by some obvious predisposition of the species, might be caused by the high densities of other species which are predisposed to aggregation.

Over-dispersion tends to increase the heterogeneity of the vegetation, while under-dispersion accompanies homogeneity. The greater the number of species with individual plants of each showing over-dispersion, the greater will be the heterogeneity of the stand. For example, in a well developed stand dominated by *Agropyron smithii* in the northern Great Plains, a high degree of homogeneity is manifest because other species do not have opportunity to aggregate, due to the intense competition of the wheat grass. But in an over-grazed stand, where the competition has been reduced and openings have appeared, mats or groups of species, for example, *Artemisia frigida, Aristida longiseta, Opuntia* spp., *Buchloë dactyloides* and *Bouteloua gracilis,* may develop and form aggregations of high sociability. The vegetation has become distinctly over-dispersed and heterogeneous. The term "index of diversity" has been proposed to designate the degree to which groups are divided into units (301).

If the sample plots are too small or if inadequate methods are used, the manner in which the plants are dispersed may remain obscure. The plot may be so small that it is entirely within a single unit of a highly gregarious species, such as *Buchloë dactyloides.* The study plots should be of sufficient size and number so as to reveal the real nature of the vegetation, and full consideration should also be given to location with respect to homogeneity in topographic and soil conditions. Data on frequency and number of individuals (see below) are most useful in determining the kind of dispersal of a species and the degree of heterogeneity of a stand. The "isonome" method, i.e., lines plotted on a chart to show equal frequencies of plants of separate species in a community, can be employed to indicate the pattern of dispersal of both the original invaders and the increase in population. Some species have shown a continuity of isonomes from one stage of succession into succeeding stages (202). If there is insufficient time for detailed work, the listing of the species in a stand and recording the sociability of each according to Braun-Blanquet's (18) scale are useful.

Somewhat related to the sociability of individuals of species is the association of two or more species in a community. *Stipa comata, Koeleria gracilis* and other species are commonly associated in the prairies; *Agropyron smithii* and *Distichlis stricta* on somewhat saline soil; *Agropyron spicatum* and *Poa secunda* in the lower grassland zone in southern British Columbia (267); *Poa pratensis* and *Trifolium repens* in many pastures; etc. This association is due to similarity in the tolerances of species, similarity in geographic ranges, differences in biological equipment as shallow and deep root systems so that excessive competition can be avoided, dependence of one species upon another for shade, food as in parasites, etc. One species of a community therefore often has indicator significance for the presence of other species. As environmental conditions change, the associated species also change (122). Dominant species indicate other dominants as well as subdominants, and the latter indicate others of their own kind. A species growing as a dominant would usually have different associates than when growing as a subdominant in a different community; for example, *Agropyron spicatum* in western North Dakota, where it is near the eastern limits of its range, has a number of different associates, among them *Muhlenbergia cuspidata, Carex filifolia, Bouteloua gracilis* and *Eurotia lanata,* than when it is a dominant as in British Columbia (267, 298). The absence of some associated species may have especial indicator significance, such as severe competition, infection by fungi and certain unfavorable environmental conditions (43). Methods have been devised for testing forage plants in mixtures, rather than singly, because the former is the more normal situation in pastures (139).

In the quantitative measurement of ecologic association between species Dice (73) proposed the "association index" which was obtained by dividing the number (a) of random samples of a given series in which species A occurs into the number (h) of samples in which species A and B occur together; association index B/A $= h/a$, and the reciprocal index is association index A/B $= h/b$. "For example, if species A should occur on a total of 40 sample plots ($a = 40$), and if species B should occur together with A on 30 of these plots ($h = 30$), then the association index of species B with species A (B/A) would be $30/40 = 0.75$. This index shows that on three-quarters of the plots on which species A occurred, it was associated with B. . . . the association index may differ depending upon which species is used as the basis of comparison". In the above example species B was used as the base; if species A were used, the association index would be 1.0. "Frequently one species is dependent upon another, without there being any reciprocal tendency. It is believed that the association indices may sometimes be of value in discovering such unilateral tendencies". At least 100 samples should be taken for these comparisons. The amount of association between three or more species can be secured by an expansion of the formulas used for two species, but this is rarely practical. This method was based evidently upon the sampling of animals, but it should be applicable also to plants.

QUANTITATIVE

Quantitative characteristics of the community, in contrast to the qualitative, are those which can be measured or counted. They are discussed under the following categories: *a*) number of individuals; *b*) cover; *c*) height; *d*) weight; *e*) volume; *f*) frequency. While quantitative data are essential in grassland ecology, they do not displace detailed descriptions of plant communities nor of the species.

Quantitative characteristics have often been estimated because actual counting and measuring are time-consuming, and for this purpose various scales have been devised. Estimations over large areas are often considered more reliable than intensive measurements on a small number of sample plots (18, 72, 227, 77), and they are especially valuable for preliminary and extensive surveys (224, 218, 258, 241). Such surveys give important general impressions of the grassland and aid in orienting the problems of major importance. "They are not an accurate or reliable means of evaluation and should serve only as a background for developing research programs designed to solve the major problems of an area or region" (2).

The design of an investigation involves selection of characteristics for detailed study, and choice of methods will depend upon the nature of the problem, type of vegetation, time available, facilities, etc. Recently the advantages and limitations of 16 methods were reviewed (2). The report of the joint committee of the American Society of Agronomy, American Dairy Science Association and the American Society of Animal Production (137) on characteristics and procedures has been widely used, and the following excellent statement on planning is quoted:

"It is not desirable to standardize every technique to the point that an investigator accepts it blindly as a stereotyped pattern. Such a practise prevents progress and improvements in methods of research. Many minor details of technique should be left to the good judgment and experience of the individual investigator to decide, and to make application to local conditions. Plants, soils, rainfall, day length, altitude, latitude, temperature, all cause variations which cannot be fitted to a completely standardized plan, but which require observation and the judgment of the local investigator.

The first principle of research is to attempt to hold conditions of an experiment constant, insofar as this is practical, in order to measure a single introduced variable at a time, and either to measure or eliminate all other differences between experimental groups or procedures. Notes of all details should be recorded permanently at the time, and any change of technique written down, together with the reasons therefor.

In publication of pasture research, these points should be stated specifically by the investigator, so that others may be free to give a different interpretation should they feel justified in so doing" (137).

Keller (139) discussed designs and techniques for testing pasture plants growing in competition with plants of other species, instead of growing them in pure stands, to resemble more closely the mixtures found in pastures. Some experimental work indicates that the factorial design was more efficient in the use of facilities and provided data which had wider application than a series of single factor experiments (144).

In either natural or domesticated grassland the area as a whole can be made the unit of study, or plots can be laid out in it, and sample areas may be located within the plots or within the entire area. The plot ("sampling area", "sample plot", etc.) is a measured part of the stand, as homogeneous as possible in vegetation, soil and relief. Usually several plots are used in a stand. Both the plots and the sample areas may be distributed as follows: *a*) selected locations for special study, or as typical of the stand; *b*) systematic arrangement; *c*) random arrangement. The term "minimal area" designates the smallest area of a community which contains an adequate representation of the stand. When one plot is used as the unit of investigation of a stand, its size should be at least equal to that of the minimal area, and it should contain the characteristic number of species. The size of the minimal area has usually been determined at the point where the species-area curve shows a pronounced flattening.

Cain (26) described a modified method for determining the minimal area from the species-area curve, in which sampling is considered sufficient when an increase of 10% in the number of samples is accompanied by an increase of only 10% of the total number of species. Later Cain (28) used the average increase of new species per additional sample units. Ashby (8) stated that Cain's methods are still not really objective because the minimal area is selected objectively on the basis of the observer's field experience. But Cain's and the methods of others do give a valuable criterion for determining the size of the minimal area. Ashby states further that Cain's attempt to define an adequate sample area brings into relief the limitations of the statistical method in plant sociology, namely, that the samples should be randomly distributed, and the plants being sampled should be randomly distributed or in randomly distributed groups. The ecologist is compelled to transgress both of these limitations, partly because neither the microclimatic nor the soil conditions of the plant community are distributed at random. Vestal and Heermans (272) stated that the determination of the size of the minimal area at the point of flattening of the species-area curve is fallacious because this point is different if the scale of either variable (number of species, area) is changed, and if a graphic method is to be used, the correct ratio for the scales of these two variables must be worked out. They suggest the term "reference area" instead of "sample area" in order to avoid the implication of being primarily concerned with sampling procedures and because the areas being studied intensively may later serve as a basis for comparison with other types of vegetation. They suggested four kinds of reference areas: *a*) effective plot size for species-number, *b*) minimum area for assignment to type; *c*) area definitive for composition; and *d*) area for form of stand.

In alpine vegetation in Wyoming the minimal area of a single plot per stand, on the basis of number of species and areas of plots, was about 32 square meters, but if scattered plots were used the minimum size of 0.1 sq. m. and at least 20 per stand would be required to calculate satisfactory frequency percentages and coverage averages (28). A distinction has been made between "minimal area" and "minimum-quadrat area", the former referring to a single sample area per stand, the latter to several scattered quadrats per stand (25, 30). In the Festucetum halleri community of the Caricion curvulae alliance, Braun-Blanquet (18) determined the minimal area at 20 to 25 sq. m. (with about 52 spp.), while in the Curvuletum community, in the same alliance, it was 10 sq. m. (with about 30 spp.).

In a recent careful study of phytosociological relationships Penfound (198) concluded that the size of the sample area can not be predetermined if frequency, density (number per unit area) and cover are to be studied, and that the size is relatively unimportant if frequency is omitted, since the size of the sample area does not affect density or cover relations. The number of sample areas to use depends on the size of the sample area, and when this has been decided, the number to use can be determined by the break in the species-area curve, but, on the basis of his observations, Penfound concluded that the number should be at least ten times the number indicated by the break as a proper quantitative sample.

Various sizes and numbers of plots, depending largely upon the purpose of the studies, have been used. In a study of biotic communities in the northern desert shrub in Utah, 0.25-sq. rod plots were used (87); in artificial reseeding experiments in "closed" communities of *Bromus tectorum* and depleted *Artemisia tridentata* in the Great Basin, the size ranged from 0.01 acre to ten acres (223); in testing mixtures for irrigated pastures plots, 22.5 sq. ft. replicated 12 times for 32 mixtures, and plots 25 sq. ft. replicated four times for 36 mixtures (140); and in a study of the natural revegetation of abandoned plowed land in Colorado, plots of 25 sq. ft. located inside 20-acre exclosures and in grazed pastures

(58). In pasture studies in Nebraska plots of about ten sq. rods and 30-sq. ft. exclosures were used (284, 288). Sample areas located in plots yielded data of greater reliability than scattered sample areas not in plots in western North Dakota (114). In a well laid out pasture experiment in eastern North Dakota, each of two 9.36-acre pastures was divided into plots ten by ten rods in area. Within each plot four sample areas, 14 by 31.375 inches, comprising 0.00007 acre, were distributed at random, making a total of 64 sample areas per pasture. One permanent exclosure plot, two by two rods, and a number of movable cages, one m. sq., were also used in each pasture (297). In a carefully planned study of a used (nearly in native condition) and a not used grassland mesa in western South Dakota, four-acre plots, 88 by 220 yards, divided into 15 subplots, and two sample areas of one m. sq. per subplot, were used (151). The relation between the frequency index and abundance was studied in southwestern U. S. with plots 10,000 m. sq., and sample areas of one and 0.1 sq. m. within them (165). The number of sample areas needed in each plot to achieve a specified degree of accuracy depends upon the area of the plot and the nature and distribution of the species composing the vegetation (173, 198).

The transect, such as the line interception method (32, 33, 234), is inherently an elongated sample area. In this method about 16 lines, each 50 or 100 ft. long, were located at random in uniform plots into which the stand had been divided. The size of the plot was not important when 16 or more sample units at least 50 ft. long were used per plot. The point-observation-plot technique (253) has been used rather extensively in surveys of large areas; for example, in studying the influence of unrestricted grazing in western Utah in one valley, 920 plots, each 200 sq. ft., spaced at intervals of 132 ft. on a transect 23 miles long, were used (251). The same method was employed on the vegetation of the Fort Worth Prairie in Texas, with 169 100 sq. ft. circular sample areas spaced at 350-ft. intervals on a transect 34 miles long (78). Sample areas of 100 sq. ft. at intervals of 350 ft. on a transect 76.5 miles long were used in investigating the Western Cross Timbers in Texas (79).

Carefully selected plots have been employed effectively to determine in a general survey the extent and general nature of vegetation (267); the forage preferences of livestock (57); the course of succession after plowing or drought (299, 300, 282, 283), in newly seeded pastures (110) and following burning (111); to record changes in the course of time (4); etc. The systematic arrangement of sample areas has been used to determine the effects of different systems of grazing upon a western wheatgrass community in Colorado (120) to ascertain the characteristics of the major grassland communities in western North Dakota (122), to determine the effect of isolation transects upon the intensity of grazing by cattle (112), in comparing the plant composition of ridges and furrows in abandoned sugar-cane fields in Louisiana (199), etc. The random distribution method has been relied upon by many (76, 266, 297, 30). In a recent text on range management (258) the development of refinements in grassland ecology is indicated by the discussion on the need of plots in range management and research, and the advantages of systematic and random arrangements as compared to the selection method.

In a comparison in the field and in the laboratory (with discs) the two methods were found to have about equal accuracy in communities where the individuals were all of the same size (an unnatural condition), but the transect method required much less time (11). In communities in which the individuals varied in size, as occurs in nature, the transect method was more accurate in measuring cover, due probably to the greater linear extent of the transects. On the basis of laboratory tests "the per cent of the line covered in the transect samples was a considerably more accurate indication of the true areal coverage in the entire assemblage than was the per cent of the area covered within the quadrat samples". In testing results of planting grass to control erosion dunes, plots of 0.1 acre, at least four times as long as wide, with three mil-acre sample areas per plot, were used (21).

NUMBER OF INDIVIDUALS (ABUNDANCE, POPULATION DENSITY). In an exact sense "density" refers to the number per unit area, and by dividing the measured area by the number of individuals the average area occupied by an individual can be determined. The average distance that individuals are apart can be determined by taking the square root of the average area. The reliability of the average increases as the number of sample areas increases. Averages alone, however, are inadequate for many purposes in describing the community and should usually be employed with other characteristics, especially frequency (76, 118, 122). The term "density" has been used in range surveys to indicate the ground covered by vegetation (232, 238, 253, 258), but it should be used more precisely in the sense indicated above.

In attempting to deduce a general law as to how abundance is distributed among species, four meanings of abundance (commonness) and rarity have been recognized (209); *a*) global abundance, the total number of living individuals of a species; *b*) local abundance, the total number of individuals living at a certain instant on an area; *c*) relative abundance, the ratio of the number of individuals of one species to that of another; *d*) sample abundance (observed abundance), the number of individuals counted in a sample. Prát (208) stated that Bail's M-concentration concept regarding the maximum concentration of organisms in a given space applies not only in bacteriology but also to organisms generally, including the spacing of plants in agronomic practises, and even to man. In experimental work to control the erosion of dunes on the north Pacific Coast, density was considered the product of spacing between hills and the number of culms per hill (20, 21). "When sand accumulation and cost of planting were considered together, a density of 24 × 5 [inches] was most efficient on sites of moderate severity. A density of 18 × 3 was efficient only when fertilized. Higher densities, 12 × 5 and 12 × 3, were effective but not efficient unless fertilized. Spacing was more important than culms per hill in determining the influence of density on production. The number of culms per hill was more important than spacing when density and erosion control as expressed by sand accumulation were correlated".

"Density values may or may not have much significance in delineating the relative importance of species in a community. If the species are similar in size and spread the number of individuals per unit area may be a very important criterion. On the other hand, the relative size of individuals may be very different even among codominants, especially in herbaceous communities. Often it is very impractical and sometimes impossible to distinguish individual plants in the field. Since different plants, even in a given stratum, may vary greatly in volume and since the identity of individuals may be difficult to determine under field conditions, it is obvious that, upon occasion, density may have little phytosociological significance. In our investigations we found that the density relation among species did not change with the quadrat size, number of species, or total plant cover. These facts suggest that density is a truly quantitative condition and is a more reliable one than frequency" (198).

The term "percentage abundance", or "relative abundance", has been used to indicate the relative proportion of units of each species in a stand, but this usage appears valid only when the plants have similar life-forms and are therefore fairly comparable, which is rarely the case (119). "Percentage frequency" has also been used (266, 72), but "frequency" is not very exact in this connection. According to the more common usage, "frequency" should be reserved to indicate the degree of dispersion of individuals of a species in a unit area, or the proportion of the total sample areas in which a species occurs.

One of the difficulties in applying this characteristic is the determination of what constitutes a plant unit, but routine procedure has been established (71, 115, 134, 155). Often it is easier and more precise to measure the area occupied by some kinds of life-forms, such as mats or bunches, than to attempt to count tillers. "Results based on actual separations of forage from randomly selected areas are most accurate. However this method of obtaining data is slow, tedious, and expensive and has largely been abandoned in favor of less accurate procedures which are more rapid" (2). Various estimation scales have been used (18, 114, 284). Scales may combine two and possibly more characteristics, such as abundance and frequency (114), abundance and foliage cover (4). "The use of a scale of classes rather than actual percentages is justified by the fact that the getting of actual percentage is a difficult problem. It is relatively easy to say that a given species covers 1/4, 2/4, 3/4, or 4/4 of the area. This estimate is sufficiently accurate to cover the more important changes in plant population and does not require an excessive amount of time" (4). "Not infrequently mere estimation gives better results than counting and measuring" (18). In the analysis of grass veld in South Africa counting was objected to because of the great difficulty in deciding on the plant unit, the impossibility of taking turves to the laboratory for dissection and the tediousness of working numerous samples required by the heterogeneity of the vegetation. So it was concluded that the results obtained would not be commensurate with the required labor and time (295). But neither are estimates highly reliable, due to the heterogeneity of the vegetation, changes in composition from year to year, and variations in estimates resulting from the personnel factor (8, 241). Perhaps a word of caution is needed because it is so easy to take the least laborious methods; estimation methods should as a rule be used only when more precise methods can not be applied for the reason that the time available will permit use of estimates or there would be no study at all, or because the nature of the vegetation prohibits the use of more precise methods, which is rarely so.

Counting the number of individuals according to species is usually indispensable in research on reseeding, burning, chemical control of weeds or poisonous plants, reduction of weeds in succession, etc. In southern Utah if *Bromus tectorum* can be reduced from about 570 plants per sq. ft. to about 50 for one season, perennial grasses, such as *Agropyron spicatum, A. inerme, A. cristatum, Poa bulbosa, P. ampla, Bromus inermis, B. carinatus* and *Arrhenatherum elatius,* can succeed when artificially seeded (132). Abundance, and frequency also, were used effectively in showing differences between prairie relics and mowed or grazed prairie in north-central Missouri (76); and abundance has been much used in studying the effects of drought (221, 291, 299), of grazing (120) and of clipping (127). The number of stalks grazed compared to the number not grazed on sample areas has been used as a method of determining range utilization. One or a few single-stalked species are selected for counting. This method has been criticised on the basis that some stalks are grazed closer than others (258, 195). One difficulty or deficiency is that too often only one kind of measure is relied upon, and, since an insufficient number of characteristics are measured by it, the one criterion is criticised as inadequate. If it were combined with other measurements, as of height and weight, the number of stalks would be valuable.

COVER (AREA OCCUPIED, DECKUNGSGRAD). "Cover" refers to the herbage area, or spread, the area occupied by leaves, stems and inflorescences as viewed from above; "basal area" to the extent of ground actually covered by the crown, or the area penetrated by stems, readily seen when the leaves and stems are clipped at the ground surface, or the area occupied by the vegetation in a horizontal plane at one inch or more above the ground (115, 119, 232, 236, 164). The areas occupied vary according to the life-form or growth form of the species, *e.g.*, the maximum spread of *Aristida longiseta* was 1666 cm. sq. per sq. m. at a height above one inch, while of *Buchloë dactyloides* it was 505 cm. sq. at a height of zero to one inch (119). Many ecologists have pointed out the importance of plant cover. "Cover is probably the most suitable ecological expression for recording change" (64), but this would not hold true for single- or few-stalked plants, such as *Agropyron smithii* and many forbs.

Penfound (198) stated that "herbage area" has been used only to a slight extent in community analysis, and that "basal area" and "dry weight" have been favored in determining dominance, but, since it is easier to determine herbage area than basal area or dry weight for ground, herbaceous and suffrutescent strata, it is surprising that phytosociologists have not used herbage area more. He stated that herbage area can be estimated with considerable accuracy, but others disagree (241, 8). According to Penfound, herbage area is a more important character than frequency or abundance (density) because it can be estimated in percentage and is not affected by the size of the sample area. He felt that his conclusions were supported by Bauer (11) who thought that coverage rather than the number of plants, frequency or other quantitative characteristics determines dominance and gives character to the community, and that data on cover may be considered more valuable and significant than other data to express quantitative relations among species. This may be true, but the use of cover alone, which might result from such emphasis, can not give complete nor even all of the necessary information about a community. In regard to dominance alone the data on abundance, frequency, weight, height and qualitative and synthetic characteristics are essential.

In much research on grazed grasslands, charting or measurements have been made at one inch above the ground, the height to which many species are grazed, and this has been called the "basal area" by many (230), but this term should be restricted to the area at the ground level. Other objections to the use of herbage area are that it is readily affected not only by grazing, but also by insects, fungi, weather variations, etc. Basal area, however, is more stable under these ephemeral influences, but it is subject to more long-continuing influences. Therefore, for many objectives the measurement of the basal area gives more valid results.

The chief quantitative methods used in studying cover are: *a*) charting by hand, pantograph, tripod, camera, or traverse board; *b*) area list; *c*) point contact; *d*) line interception; *e*) mirror projection; *f*) photographic negative. Some of the estimation methods are: *a*) cover scales; *b*) ocular or range reconnaissance; *c*) point-observation plot or square-foot density. Since most of these methods have been fully described in various publications (258, 285, 18, 115), this discussion is restricted to recent applications of some of these methods and to some of the newer methods.

The tripod method of charting vegetation was used (17) on a sample area, 2.5 ft. sq. At the apex of the tripod, placed directly above the sample area, was an eye hole, and 15 inches below it and 30 inches above the ground was a piece of shatter-proof glass, fastened to the tripod, 12 in. sq. After the vegetation was clipped to a height of one inch, the bunches were outlined on transparent paper placed on the glass. This method was used for three seasons in bunchgrass communities and required a small fraction of the time needed by the pantograph or grated quadrat methods.

In the area-list method the basal areas, or areas at one inch, are measured by a ruler or special measuring instrument, such as the listing square (135), the area rule (194) or the densimeter (65). The density-list method (percentage area) is similar to the area-list method except that there is more estimation and less measurement in the former, and the data are recorded usually as percentages instead of in sq. cm. or inches (5, 71, 72, 82, 88, 119, 169, 230, 179, 246, 267). While the density-list method saves much time and a high degree of accuracy may be attained by skilled personnel, it is not suitable for regional surveys (64). Estimates of percentage cover in pastures by different observers are subject to wide discrepancies (295). The "chief objection to the method lies in the apparent inability of the observer to estimate consistently the percentage of area covered by each species in different types of swards. The method fails if the vegetation is sufficiently tall to be displaced or affected by placing the frame on the sward" (266). In conclusion, wherever the vegetation permits, the area-list method, or the point-contact method, is preferable to the density-list method, because both of the first two methods are more objective. The point-contact method (point-quadrat method) (153) was apparently first used in the U. S. by Hanson (114) in a comparison of methods in the analysis of vegetation in western North Dakota. It was also used to measure herbage cover in determining the characteristics of major grassland types in this area, employing 300 points per stand of two to 20 acres. Du Rietz (77), Davies (71) and Fenton (89, 90) called the attention of British and European workers to this method, and in So. Africa West (295) concluded that it "possesses the virtue of speed, making it possible to cover a large area fairly thoroughly in a reasonable time. In addition its results possess a quantitative value. . . . A point very much in favour of the method is the elimination of the personal factor". The procedure was modified (266) by setting the pins in the frame at an inclination of 45° instead of vertically, giving the method certain advantages as being more easily used, covering a greater area per reading and increasing the accuracy. When a correction factor is used some investigators have found close relationships between the point determinations and dry weight percentages (7, 6, 75), but other investigators concluded that inaccurate results would be obtained by using a constant for correcting the point contact data (243) because of the varying ratios secured between point and weight data at different times of the year. This is to be expected because the methods measure two different characteristics, area and weight (114), and usually data on different characteristics, even for the same species, are not comparable (64). In some studies apparently too few readings, as ten points in a 250-sq. ft. plot, were taken for valid results (220). The inclined-point method proved well suited to investigating the effects of soil acidity and available phosphorus on population changes in mixed Kentucky bluegrass turf (180).

Crocker and Tiver (64), after a comparison of methods, selected the point-contact method for grassland surveys in the South-East of Australia "because it incorporates so many desirable features, and seemed to be so readily applicable to large-scale work. Its most valuable features are: *a*) it permits quantitative determination of botanical composition in terms of cover, which is probably the most suitable ecological expression for recording change; *b*) it is objective and more rapid than other methods of equal reliability and objectivity; *c*) it provides for randomisation and ample replication of sampling; *d*) it also permits close examination of all, or almost all, species present and gives plenty of scope for observation; *e*) it does not depend entirely on random distribution of species for its usefulness; and *f*) it does not in any way interfere with the vegetation. 300–500 point samples per unit area (field or paddock) [14–150 acres] are usually sufficient for a reliable analysis of a pasture in survey work. Provided edaphic variability is not great, uniformity of treatment is more significant than most other factors, especially area, in determining botanical variation, and the size of the paddock is not very important for the number of point samples required". Species of markedly different growth habits, such as thistles which in late spring had already grown to a height beyond the possibilities of this method, were studied by the count-list or other methods. The optimum height of the vegetation for the use of the point-contact method was considered to be four to six inches, and sampling should be avoided during a high wind in order to reduce the error.

Since Levy introduced this method, it has been widely used, found most serviceable and is becoming one of the standard methods of measuring cover. It is rapid, accurate and quantitative, especially in grassland up to eight inches tall.

The line-interception method (32, 33, 129), a modification of the line transect, lists and measures the linear extent of the basal areas of all plants that are intercepted by the vertical plane of randomly located lines of equal length. When the ground cover is five to fifteen %, lines 50 ft. long were used; with ground cover of 0.5 to 3% the lines were 100 ft. long. Not less than 16 sampling lines should be placed at random in each area to be sampled, regardless of the size of the area. Shrubs and half-shrubs were measured separately from the grasses and forbs, projecting the line vertically to intercept the maximum spread of the crowns. The linear extent of each plant is recorded in the field to the nearest 0.01 ft., and from these measurements the percentages of ground covered and the relative composition of the vegetation is calculated. The proper length of the line must be determined for various types of vegetation. Close agreement was found in the data secured from the line-interception and permanent chart quadrat methods (32, 3), and both of these methods were used in a study to determine the condition of mixed grass ranges by the proportion of perennial grasses present (34).

Crocker and Tiver (64) found the line-interception method valuable in the quantitative analysis of fairly open grasslands, but in dense vegetation it had limited application, due to the difficulty of recognizing individual species. The line-interception method was modified for use in the sand-dune-sage type of vegetation in the southern Great Plains (234) by using a belt ten cm. wide at a height of 44 inches for measuring the peripheral spread of shrubs, primarily *Artemisia filifolia,* and a strip one cm. wide on the ground for the cover of grasses, sedges and forbs. This method was carefully tested for accuracy and reliability with 3,729 lines, each ten meters long, on 20 pastures (190). The composition of the sand-dune-sage type with a total cover of 34.3%, according to this extensive study, was: *Artemisia filifolia, 79.2%*; *Bouteloua gracilis,* 6.34%; *Sporobolus cryptandrus,* 5.25%; other perennial grasses, 2.42%; annual grasses, 0.9%; other shrubs, 4.88%; and all forbs, 0.99%. A total of 50 grass species and 216 species of forbs and shrubs were recorded. In this vegetation type it was concluded that "Meter quadrats are too limited in scope to represent accurate sampling areas for highly variable brush-infested vegetation and slopes. A pantograph table would have to be located above the brush and too far from the ground surface to permit strictly accurate readings. The line transect appears to be admirably adapted to the situations encountered on the experimental range. It represents a desirable combination of speed and accuracy. Each line provides a sample through and between several shrubs and over a constantly changing topography". The line-interception or the belt-transect methods outlined above have been used in an extensive test to determine the best time for cutting prairie vegetation to obtain high quality of hay and to improve the grassland (9), in brush vegetation in California (11) and for determining basal area of plants in grasslands in Montana (304). These methods, in conclusion, appear to be most suitable in vegetation where shrubs are intermixed with grasses and forbs, and not so well suited to dense grassland types.

In the photographic negative method (128) bent grasses given different fertilizer treatments in pots were timed at the end of six weeks to a uniform height and photographed at night, using a constant source of light. The negatives were very dark, due to the white quartz sand in the pots, except for the area occupied by the grass. Diffused light passed through the negatives was measured in foot candles with a Weston photo-electric cell, and these readings were used as a direct measure of the relative amount of coverage.

Various modifications of scales for estimating cover described by Braun-Blanquet (18) have been proposed (198, 200). Vestal (271) reviewed various scales and proposed an unequally ten-parted (and a cognate five-parted) scale with the aim of finding a more smoothly graduated scale. The proposed scales could be used with the largest steps at the bottom or at the top, as applications indicated. However, "Since gradings by means of scales are to a degree subjective and arbitrary, findings from their use are usually indicative, rarely conclusive. One can better judge which scale to apply to a given set of data by applying more than one, and comparing the results" (271).

An excellent account of the ocular or range reconnaissance and the point-observation-plot or square-foot density methods of grassland analysis by estimation is given by Stoddart and Smith (258). The latter has been widely used during the past ten years in range investigations (59, 196, 217, 218, 251). Smith (241), in a noteworthy study on the reliability of range vegetation estimates, concluded that "These studies do not show density estimates to be a highly reliable measure of range value since 'high' estimators found ranges to have almost twice the grazing capacity found by 'low' estimators, even after a week of intensive training. A significant difference was found between individuals and also a given individual was found to have significant inter- and intra-daily variation. In addition, plants showed great variations in yield from a unit of density both within and between species. The results of this study support the current tendency among range technicians to rely less upon density estimation as a means of determining grazing capacity of ranges, and more upon comparison with ranges of known productivity and a general ecological analysis of plants and soil followed by percentage adjustments in current stocking".

Crocker and Tiver (64) stated that estimation methods have been widely used in ecology and that they can reach a high degree of reliability, depending upon the individual skill of an observer, but they are less and less reliable for regional surveys which demand the employment of a large number of field men. "Furthermore, the dynamic nature of the pasture complex makes the application of objective methods doubly desirable". Ashby (8) objected even more strongly to estimation methods: "Each of these four characteristics [fidelity, abundance, cover, frequency] can be measured objectively and expressed quantitatively, and qualitative studies of them are, or ought to be, as out-of-date as qualitative studies of respiration. There is adequate proof that the older subjective methods are unreliable".

There is no question about cover being one of the most important of community characteristics, but this does not justify its use to the exclusion of other characteristics. The most valuable methods are one of the charting methods when permanent records of areas and locations of each plant in the sample area are required, and the area-list, point-contact, or line-interception methods, depending upon the nature of the vegetation. Estimation methods should be used only in preliminary indicative work and can not be considered as substitutes for quantitative methods.

HEIGHT OF PLANTS. Height is a good index of vigor and can be used as a criterion of the suitability of the environmental conditions for the growth of a species. Since there is a good correlation between height and weight measurements of the rate of growth, curves are frequently based on height data instead of on weights which are more difficult to secure (145). Measurements of height, width or diameter of parts should be made as precise as possible by considering a large number of individuals.

The average length of the leaves on one plant selected as of average height in each row of each plot was used in studying the effects of competition by *Artemisia tridentata* upon 17 species of range grasses (222). In early spring *Agropyron cristatum* had reached a height of four inches under intense competition and seven inches where there was no competition; in *A. smithii* the measurements were 15 and 17 inches, respectively. Grasses without brush competition attained the eight-inch average height on April 26, but under reduced and intense competition the respective dates were April 29 and May 3.

The foliage height and the working and maximum depths of the root systems show relationship (287). For example *Buchloë dactyloides* with a foliage height of 5.5 inches had working and maximum depths of 12 and 20 inches, while with a foliage height of three inches the working and maximum depths were five and nine inches. The width of the third leaf, near the middle, showed relationship also with the diameter of the roots at a depth of six inches in six grass species.

Flower stalks may be cut at the close of the season and taken into the laboratory for making precise measurements. The average length of 100 stalks of *Agropyron smithii* from a deferred and rotation pasture in Colorado was 23 inches, while from a continuously grazed pasture the height was 18.7 inches; and for *Stipa viridula* the respective lengths were 32.1 and 24.1 inches (120). Rate of growth was also determined for six species by measuring the maximum height in the field of two to ten stalks in each of ten clumps for each species. Earliest growth began in the middle of March, was most rapid during May, and vegetative growth was completed in the latter part of June when the available soil moisture was depleted. *Stipa viridula* was less sensitive to low temperatures early in the spring than *Agropyron smithii,* and both were less sensitive than *Bouteloua gracilis, Buchloë dactyloides, Schedonnardus paniculatus* and *Aristida longiseta.* In southern British Columbia the average mature heights during the period 1935 to 1940 for *Agropyron spicatum* were 14.6 inches for the leaves and 22.1 inches for the stems; for *Stipa comata,* 5.1 and 16.2 inches; for *Poa secunda,* 2.0 and 10.5 inches; and *Bromus tectorum,* 8.5 inches for the stem only (267). In eastern Montana *Bouteloua gracilis* clipped at two cm. every two weeks during the growing seasons from 1938 to 1941 inclusive had seed stalks in 1942 (not clipped in this year) 23.7 cm. tall and basal leaves 11.2 cm. long; the plants clipped at two cm. every four weeks had respective heights of 24.4 and 13.1 cm.; those clipped at four cm. every two weeks were 26.6 and 15.4 cm.; and those clipped at the end of the season only were 30.3 and 15.9 cm. (127).

In Utah the annual herbage growth of range grasses, such as *Bromus carinatus* and *Agropyron trachycaulon,* begins 45 to 89 days before disappearance of the winter snow, and the annual growth is cyclic, due to the plant's resproductive cycle and to the influence of climatic conditions (163). Several studies have been made on the effect of precipitation received during the current and previous seasons upon height growth and area of tufts (181, 157, 120).

One of the important practical applications that has been made of height measurements is the determination of range readiness in the spring. Several criteria have been used, such as the time of appearance of first flowers in some species, appearance of flower heads of certain grasses, and height of foliage of grasses, ranging from two to ten inches, depending upon the species (231, 258, 61, 60). Considerable research has been done on methods of relating height utilization to weight utilization of range grasses, such as the preparation of tables based on weight measurements of each inch of height growth (158), use of converting factors and curves to transfer height to weight measurements (52, 62, 217), formulation of a scale for field use (159), and comparisons with other methods to determine degree of forage utilization (41). The results of these investigations, many of which are contradictory, indicate that the relationships between characteristics are most complicated. It appears only natural that direct relationships would not occur consistently because the response of a plant in one characteristic to environmental conditions is not necessarily dependent upon the response in some other characteristic. Since there are so many parts of plants that can vary in time and in place, in height, weight, area, thickness, chemical composition, etc., it can not be expected that simple relationships will occur. Therefore it appears that each characteristic must be measured directly, and the transfer of readings of one characteristic to another is rarely, if ever, valid.

WEIGHT OF PLANTS (YIELD, PRODUCTION). Weight is one of the most important quantitative characteristics of vegetation because increase in dry weight is probably the best single measure that can be made of growth (294). Height and area, or spread, of the herbage are important space characteristics, while weight is the quantitative expression of the structural materials, foods, protoplasm and minor substances, such as crystals, that have resulted from the metabolism of the plant, and form the basic substances for additional growth and for endurance of and recovery from unfavorable conditions. Most of the research on weight has been done on the herbage as a whole, but in recent years some work has been performed on the weight of the root system and also on leaves separately from stems. In greenhouse experiments (203) the dry weight of the roots of Sudan grass was one-third of the total weight of the plants, regardless of the stage of maturity, and addition of nitrogen fertilizer caused a decrease in the proportion of roots to tops. In New Jersey (242) the weight of roots of pasture and turf grasses showed a rapid decline between the first and sixth inches. In West Virginia (101) the weight of roots of pasture grasses was greatest in the uppermost three inches.

In estimating the amount of green forage on range lands of western U. S., it appears that the estimates based on weight are more reliable than those based on area (258, 197, 96). The weight of the forage on sample plots is estimated by species, and then the total production for the entire area under investigation is calculated. Considerable training is necessary to use this method, but the value of it is that actual weights can be used in the training and also in checking the estimates. In investigations in southern U. S. (31) actual weights of green and air-dry herbage were used from square plots of 9.6 sq. ft. with 10 to 15 plots per sub-type, this eliminating estimations.

Clipping of the herbage within sample areas to obtain green or dry weights has been widely practised, and it has been called the "direct harvest" method (182). West (295) stated that it "is the most suitable form of the Percentage Productivity Method for use on natural grassveld in South Africa". Ahlgren (2), after comparing many methods, stated: "Clipping or mowing ungrazed plots or protected areas in pastures which are grazed to obtain yields of dry matter is probably the most common procedure used in measuring the results of pasture research. The method is easy to employ, rapid, and relatively inexpensive. It is admirably suited to trials which include a large number of variables and for small plot types of studies". Many sampling procedures have been used, such as cutting with grass shears, hand sickles, lawn mowers, field mowers, and hand plucking to more closely resemble grazing. Fenced plots and caged sample areas vary from a few square feet to several square rods or larger, and they may be located permanently, annually or for short periods, depending upon the frequency of clipping. For most kinds of research the cages are moved to new locations after each clipping. Investigators have found conflicting results from clipping in comparison to those from grazing, among them greater yields from clipping than grazing, lower yields under clipping, close relationships and poor or low relationships. This is to be expected because of differences in growth habits and life-forms of grasses, grazing methods, time of clipping, etc. "On the basis of data given in the literature, there is evidence for a substantial difference of opinion with respect to the validity of results based on yields from mowed plots compared with those of grazed areas. However, most investigators have reported that results based on clipping trials which simulate grazing approximate those obtained in grazing during the first few years. There is some indication, however, that significant changes may occur in the botanical composition of grazed and mowed areas and that clipped areas deteriorate progressively in quality and productivity as time goes on. In view of these results it appears highly desirable to avoid the use of permanent sampling areas in grazing trials and to relocate cages or other sampling devices at reasonably frequent intervals. It is clear that additional information is needed relative to the value and accuracy of results based on clipping or mowing compared with those obtained in grazing trials. Until such information is more generally available, results of clipping or mowing trials can be interpreted only as being indicative of those likely to prevail when livestock are used" (2).

According to the usual movable cage method (154, 182), the difference in the weights of clipped herbage produced within plots protected from grazing and of that remaining after grazing and then clipped represents the quantity consumed by the livestock. However, when the herbage is clipped before placing the cage in a new setting, the vegetation is defoliated to a greater degree by this clipping than that which is exposed to grazing and not clipped, and therefore this method is subject to criticism (155). Another source of error occurs when growing conditions are favorable and the length of the grazing period is long, in that the quantity of grass available at the end of the grazing period may be materially greater inside the protected area than that which had been available to the livestock in the pasture (155). A correction factor was introduced "which would have the effect of allowing for differences in growth during the actual grazing period of the herbage which had unrestricted growth inside the cage and that subjected to defoliation by animals outfield during the same time". At first it was thought that the animals ate the same quantity of herbage each day, but, in accordance with results obtained by other investigators (136, 303) which showed that the daily consumption on pasture drops very materially as the quantity of available forage becomes less, the rate of consumption is in proportion to the quantity of grass available. Experiments showed that the total yield of the direct harvest method with a correction factor differed by 18.2% from that of the animal weighings, by the uncorrected method by 49.0%. It was concluded also that "the output of animal products even when large numbers of stock are used is not necessarily in all cases a good measure of the quantity of grass which has been consumed, even when, . . . the grass is of uniform quality as indicated by chemical analysis. . . . In much of the literature dealing with comparisons between animal output and that by grass clippings it seems to be tacitly assumed that animal output figures are not subject to error and any discrepancy between the two methods is deemed to constitute error of the cage method only. This is obviously wrong. Clearly each method has its own error and each contributes to the error of any difference between the two methods" (155).

Ahlgren (2) reviewed briefly nine methods for measuring the results of pasture research based upon the use of animals, and seven other methods in which animals are not used. The animal methods, all of which involve the weight of plants, are based upon profit, production of milk, cattle and sheep weights, pilot plots, total digestible nutrients, carrying capacity, palatability trials, digestion trials, and biological assays with small animals.

Periodic weighing of beef cattle, dairy heifers or sheep is less complicated than measuring the production of milk from dairy cows, but similar precautions must be taken in the selection of animals. The same class of livestock should be used throughout the experiment because the effects of different kinds upon the grassland varies (136, 172, 220). In Great Britain special methods have been developed for utilizing sheep in evaluating pastures, such as movable pens and tethering. The animal is considered an instrument for estimating or measuring the productivity of the grassland (247). Some objections to the live-weight method are the difficulty of control, the relatively small areas used and the difficulty in comparing a grassland grazed under the experiment to one grazed throughout the year under field conditions (205). The total-digestible-nutrients method is considered by many to be the best for evaluating pasturage on the basis of milk-producing dairy cattle. Procedures for this method have been described by the joint committee of the American Society of Agronomy, American Dairy Science Association and the American Society for Animal Production (137, 100, 270, 220, 143, 146). The requirements of digestible nutrients for milk production and maintenance and for live-weight increases are considered in relation to the nutrients supplied in supplementary feeds and in the pasturage.

One of the most common ways of expressing the forage value of grassland has been to state the "carrying" or "grazing capacity", i.e., the number of days of grazing that an area provides for a certain length of time. Various terms have been used, such as "cow days", "sheep days", "cattle days", "grazing days", "cattle equivalents", "cattle grazing weeks", "pasture days of grazing", "unit day of grazing". A standard "grazing day" has been proposed (66) as a mature sheep grazing for one day in the month of June in England. A day in August would be equal to 97% of this, in October 74%, in December 17%, in January 16%, and in March 30%. The number of "standard cow days" per acre (2, 146) is secured by dividing the total digestible nutrients per acre by 16, and the carrying capacity is then calculated by dividing the number of standard cow days per acre by the number of days of grazing during the season. Use, however, of grazing units without including weights of the animals or of the milk, and supplementary feeds, is not very quantitative and should be limited to preliminary trials or to studies where the emphasis is on the effect of the animal on the vegetation rather than the effect of the forage on the animal.

The only direct method of measuring the value of forage is by digestion trials, in which plants are fed to livestock in carefully controlled feeding experiments. On account of the limited amount of information available, the pasture committee of the American

Dairy Science Association reviewed the data of 45 digestion trials and suggested the following coefficients of digestibility for mixed pastures: crude protein, 75%; crude fiber, 79%; nitrogen-free extract, 80%; and ether extract or fat, 50%. If chemical analyses are lacking, the dry matter may be assumed as 72% digestible, and for dried grass with 15% moisture and 9% ash this is equivalent to 60 pounds of digestible nutrients per 100 pounds (137).

The method of biological assays, in which small animals as rabbits, guinea pigs and rats have been used in evaluating the forage, offers considerable promise because of the similarities in results obtained with sheep and cattle (260).

Much research has been conducted in the determination of the chemical composition of plants (175, 274, 12, 131, 260, 188, 191, 95, 97, 235). A number of plants have been shown to contain toxic substances, such as selenium (176, 85). Analyses are also being made in increasing numbers for carotene content (124, 273, 168, 94). A number of studies have shown the importance of organic food reserves in relation to the growth and winter survival of grassland plants under different intensities of grazing or clipping (162, 163, 256). One of the objects of the U. S. Regional Pasture Laboratory has been to develop methods for evaluating herbage by chemical analysis alone without laborious digestibility trials (94). The process of lignification, which proceeds rapidly as plants mature, is a major process in decreasing the value of carbohydrates as food for animals, so the importance of including lignin in chemical analyses has been emphasized (98, 191, 260). The portion of the herbage that is actually consumed by livestock should be analyzed for its feeding value because sheep select grass and clover leaves in preference to stems when grazing. Since this is most difficult to do, Raymond (216) suggested a method of testing the faeces of sheep for nitrogen and ash. From this nitrogen content the nitrogen in the feed can be derived.

The relative palatability of the herbage of species may be used to measure the effect of improvement practises, such as the introduction of new species, application of fertilizers, mowing of weeds, value of the various species in pasture mixtures, evaluation of new strains or varieties, and managerial practises. Palatability is affected by many factors, such as the kind of species or variety, density of the vegetation, exposure, stage of growth, soil fertility, temperature conditions, animal habits, pure stands or mixtures, presence of objectionable substances as tannin in lespedeza and coumarin in sweet clover, presence of attractive substances as carotene and high moisture content (260, 2, 137).

In conclusion, the measuring of weight is becoming more precise and useful. Fuller understanding of the comparative importance of the species in the grassland is resulting from refinements in procedure, such as movable cages, Linehan's correction factor, improved estimation methods when it is necessary to use them, biological assays, and in chemical analyses.

VOLUME OCCUPIED BY ORGANISMS. No particular advance has been noted in the use of this characteristic since the earlier publication (115). The word "volume" is occasionally used in place of "weight", especially in publications dealing with the height-weight relations of range plants. The term "weight" is more precise, and it is desirable to reserve "volume" to denote the three-dimensional space occupied by plants. Additional data on volume of plants from sample areas, in relation to data on other characteristics, are needed to enlarge our knowledge about grasslands.

FREQUENCY. Frequency is concerned with the dispersion, uniformity of distribution or ubiquitousness of the plants of a species throughout a plant community or a stand. It is measured by determining the presence or absence of a species in sample areas which are distributed as widely as possible in the stand, and the results are expressed in percentage, the "frequency index" or "percentage frequency". "Frequency gives considerable information concerning the nature of a flora, as some species with very low cover value have high frequency. For example, *Liatris* and *Petalostemum* species are colorful plants with extremely slender stems and leaves, hence little cover value, yet no one can deny that the appearance of the prairie [in Iowa] at certain seasons is dependent on their relative frequency" (4). A few ecologists had used this characteristic in a limited way (19, 207, 244), but Raunkiaer carried on extensive investigations and formulated his results into a generalization which has become known as Raunkiaer's "Law of Frequence" (213, 214, 215). Some of the first to utilize Raunkiaer's concepts in the U. S. were Gleason (103) and Kenoyer (141), while Hanson and Ball (117) were apparently the first to apply them to grazing studies. Since these papers were published, a large amount of work has been done in the evaluation of this concept and the procedures. This discussion will be limited largely to some of the more recent publications.

Frequency determinations have been demonstrated in a number of papers to be of value in grazing studies (117, 119, 76, 99, 69). For example, on an overgrazed area in north-western New Mexico the effect of protection from grazing on the prevalence of pingue (*Actinea richardsoni*), a poisonous plant, was studied from 1934 to 1942 by means of four plots, each 130 × 65 ft. in area. The frequency index of pingue declined on the ungrazed plot from 100% to 53%, on the lightly grazed plot from 100 to 84, on the second ungrazed plot from 99 to 85, but on the heavily grazed plot remained unchanged at 99 (99). *Andropogon provincialis* had a frequency index of 86% on domesticated prairie in Missouri and 100% on ungrazed prairie relics, while *Andropogon scoparius* had 99% on the former and 59% on the latter, and for *Sorghastrum nutans* the figures were 98 and 57, respectively (76).

Attempts have been made to integrate data on frequency and abundance. The usual estimation ratings, "sparse", "frequent", "abundant", etc., generally involve both the degree of dispersal and numbers of individuals in an area. A seven-class scale, integrating dispersion, density and cover, includes the following: *a*) dominant; *b*) abundant; *c*) frequent, quite common; *d*) scattered, fairly common; *e*) occasional, uncommon; *f*) rare; *g*) very rare (174). The frequency-abundance index is calculated by multiplying the frequency index by the average abundance (114). The average abundance is calculated by dividing the total number of individuals in the sample areas by the number of sample areas in which the species was present. Where the vegetation is extremely dense or where there is insufficient time for actual counting of all stalks in each sample area, the abundance may be estimated on the following scale for one-meter-square sample areas (30 per stand in mixed prairie): S = one stalk per sample area, I = 5, F = 15, A = 30, and VA = 100, or over. The maximum range in the frequency-abundance index figures is from 0.03 to 100.0. Usually only the chief dominants are above 50 or 60. *Agropyron smithii* by use of this method was found to have a frequency-abundance index of 83 in a native grassland on a mesa inaccessible to livestock in the Badlands of South Dakota, 68 on an intermittently used mesa, and 4 in a continuously grazed grassland. The indices for *Bouteloua gracilis* were 47, 66 and 100; for *Stipa comata*, 14, 25 and 2; and for *Carex eleocharis* and *C. pennsylvanica* (not segregated), 72, 32 and 1.5 (151). The index figures show in a most illuminating manner how *Agropyron* and *Carex* are of greatest importance in the original grassland and least important in the continuously used area, and the reverse condition for *Bouteloua*. In western North Dakota *Bouteloua gracilis* had a frequency-abundance index of 100 in the *Bouteloua-Stipa-Carex* community and in the *Agropyron-Bouteloua-Carex* community, but only 3 in the *Andropogon scoparius* community. Indices for *Stipa comata* in these three communities were 70, 1 and 0; *Agropyron smithii*, 8, 100 and 3; *Carex filifolia*, 28, 97 and 23; and *Andropogon scoparius*, 0, 0 and 100 (122). The frequency-abundance index is valuable in evaluating the importance of species within a single stand and in different stands.

It is well known that the frequency of a species varies on an area because of many influences (122, 109, 254, 264, 211). In a 15-acre upland prairie near Belleville, Kans., five "pure" and six mixed grassland types were found in 1940 following the "fragmentation" effects of the 1934–1940 drought period (286). In a comparison of the vegetation on ridges and furrows in a field abandoned four years in Louisiana, *Sorghum halepense* had a frequency index of 0% on the ridges and 4% in the furrows; *Paspalum conjugatum*, 22% and 4%; but with *Solidago hirsutissima* the "sole dominant", it made little difference, 84% and 98% (199).

The areas of equal frequencies of a species may be mapped (202) by connecting points of equal frequency in the community under study by lines on a chart, so that the resulting map somewhat resembles a contour map with centers of high frequency surrounded by lines of decreasing frequency. This method was found useful in reconstructing the history of the colonisation of species. Some species showed a continuity of the pattern of isonomes (lines connecting points of equal frequency) from one stage of succession to another, while other species disappeared or became too sparse to show patterns.

In an *Andropogon scoparius* community on Long Island, N. Y., it was determined that 25 sample areas, each 0.25 sq. m., were sufficient to study the frequency of the species (30). In the alpine vegetation in the Medicine Bow Mts. in Wyoming, 20 sample areas, 1 × 10 dcm. each, were used per stand, and *Arenaria sajanensis* and *Selaginella densa* showed the highest frequencies, 95% and 75%; more showy species, such as *Sieversia turbinata,* only 45%; and *Rydbergia grandiflora,* while present in every stand, was not found in any of the total of 100 sample areas (28). "Plot studies serve to direct one's attention to less attractive but more important plants in a community, such as *Selaginella densa,* in this case".

According to the "percentage-absence" method, the density of a species is proportional to the number of sample areas in which the species is absent. The "absence index" equals 1.0 minus the frequency index (14, 15). Ashby (8), in a critical reassessment of statistical ecology, stated that the "absence index" is a useful tool for following changes in the composition of small homogeneous plots (a few hundred square yards), but it is not so suitable for larger areas. Individuals may be randomly distributed within groups which are not randomly distributed, and individual plants may be over-dispersed but occur in groups that are randomly distributed. He thought that the distribution of each species can be summarized by the four characteristics: fidelity, abundance, cover and frequency. The most noteworthy advance in statistical ecology in the 11 years preceding 1948 was the knowledge that individuals are not as a rule normally or randomly dispersed, and that over-dispersion is much more common than under-dispersion. Cole (51) preferred the term "contagious distribution" for empirical distributions of organisms, whatever the cause of departures from randomness, the ecological implication being that the presence of one or more organisms in a sample area influences the probability of other organisms occurring in the same sample. Blackman and Ashby prefer "over-dispersion" to "positive contagious distribution" and "under-dispersion" to "negative contagious distribution". Dice (74) agrees that organisms living in nature are only rarely distributed at random, and concluded that the denser the population the higher will be the frequency, but the relationship between population density and the frequency index is not directly proportional. Preston (209), however, stated that "on the average there will be a definite relation between commoness [abundance] and ubiquity, and it is easy to pass from one to the other". He revised the boundaries of Raunkiaer's groups as follows: A) 2-22%, B) 22-42%, C) 42-62%, D) 62-82%, and E) 82-100%. He also suggested a change in Gleason's (104) statement on Raunkiaer's Law. Gleason's sentence is: "Raunkiaer's Law is merely the expression of the fact that in any association there are more species with few individuals than with many; and that the law is most apparent when quadrats are chosen of the most serviceable size to show Frequency, and that it is obscured or lost if the quadrats are either too large or too small". According to Preston it should read "in any association there are just as many very rare species as there are very common ones, but species of moderate abundance are vastly more numerous than either. In a small sample, however, there will be more species with few individuals than with many, because those with many are excessively common ones, and these species are few". Preston also prepared a chart which, in comparing data, gives the transition from one size of sample area to another size, which is intended to meet the statement that data made with different sizes of sample areas are not comparable (226).

According to Penfound (198), frequency is apparently the most artificial and least important of three community characteristics (frequency, density, cover); it has been used entirely too often and for too many purposes in the past two decades, and should be employed much less often in the future. It is hard to reconcile this statement with another one that an important reason for getting frequency data is to delineate the dispersion of a species in the stand. West (295) wrote that "Raunkiaer's Frequency Index Method is of considerable use, . . . as a rapid and approximate means of determining general floristics and describing vegetation", and that this is one of the four most important methods to use in analysis of grass veld in South Africa.

Concluding, the distribution of the individuals of a species in a stand is an important community characteristic, and unless data are given on it the community has not been completely described. The frequency index has proven to be a valuable method for comparing different types of vegetation, different treatments of the vegetation, changes in the same community, the effects of micro-topography, and in evaluating the importance of species in different communities and at different times within the same community.

SYNTHETIC CHARACTERISTICS

In contrast to analytical characteristics, which are studied by measurements and observations directly on plants in the stand, synthetic characteristics are qualities of the vegetation derived from the data of one or more analytical characteristics from one to several stands. They may be considered as abstractions or generalizations, resulting from analyses and combinations of field data. The chief synthetic characteristics treated in the literature are presence and constancy, and fidelity (18, 183, 24), but dominance and density have also been included (115). It appears desirable to include dominance with the first two under synthetic characteristics, but density should be considered in the sense of numerical abundance per unit area as a quantitative analytical characteristic.

PRESENCE AND CONSTANCY. "Presence" denotes the degree in which the same species occurs in different stands of the same kind of a plant community. The difference between "presence" and "constancy" is that in the latter definitely delimited areas are used, while in the former the only limitation on dimensions is that it should be at least equal to the "minimal area" (the smallest area which contains an adequate representation of the species occurring in the type). Use of uniform measured areas in each kind of vegetation leads to greater precision, and the data can then be analyzed statistically. Minimal area has been determined by use of the species-number: area curve, the point at which the curve begins to flatten, denoting the proper size to use, as ten sq. m. in the *Caricion curvulae* alliance, and 20 to 25 m. sq. in the *Festuca halleri* community. The flattening of the curve occurred with about 30 species in the former, and 52 in the latter (18). This characteristic has been widely used in Europe and to some extent in the U. S., although not designated as such (43, 289, 268). Its use is increasing. Since it is so closely related to fidelity, additional examples of more recent use will be given below.

FIDELITY (GESELLSCHAFTSTREUE). "Fidelity" refers to the degree in which a species is restricted to a particular kind of community. "Constancy" and "presence" deal with the occurrence of the same species in different stands within the same kind of community; "fidelity" deals with the occurrence of species in different kinds of communities. "Fidelity", then, is to some extent a measure of the ecological amplitude of a species. More use of this characteristic has been made by European ecologists (18, 184) than by the American, but the value of "fidelity" in community descriptions is being increasingly recognized (183, 24, 27, 30, 53, 115).

The degree of constancy and fidelity can be shown by means of charts, such as the 57 more prevalent species in nine vegetation types in western North Dakota (122) where the species of low fidelity and considerable abundance were *Agropyron smithii, Stipa comata* and *Carex filifolia,* while species of high fidelity and low abundance were *Stipa spartea, Bouteloua curtipendula* and many forbs. In the alpine vegetation in Wyoming 16 of 32 species (omitting grasses and sedges) had constancy determinations of 60% or more, and 34 out of 56 species (omitting grasses and sedges) had presence values of 60% or more, a dozen or more species being very important in this community (28). Fidelity may be used as "an expression of the completeness with which a given species is identified with a plant society" (4). *Astragalus caryocarpus, Viola pedatifida, Hedeoma hispida* and *Pedicularis canadensis* were fairly common on the upland prairies in Iowa; but the first two are a better criterion of the prairie than the other two because *Hedeoma* grows readily as a weed in dry soil and *Pedicularis* is a common woodland species farther east. Somewhat rare species of high fidelity are the first to disappear when the prairie is disturbed and may be the most difficult to re-establish, "These exclusive species may well be regarded as the indicators of highly developed prairie; an assemblage of the typical prairie grasses might be considered prairie, but a truly fine prairie includes these exclusive species".

Data given in various investigations (267, 277, 70, 254) can be used to determine degrees of presence and fidelity for communities in different regions, and thus the similarities and differences among them become readily apparent. For example, the grasslands of southern British Columbia resemble strongly those of eastern Washington, Oregon, Idaho and northern Utah (267), as indicated by the presence and abundance of *Agropyron spicatum* throughout the entire area and by the common occurrence of *Artemisia tridentata, Chrysothamnus nauseosus, Balsamorhiza sagittata* and other species. Egler (81), in order to show the relative importance and distributional relations of species in the Berkshire Plateau vegetation in Massachusetts, used "presence" to designate the relative occurrence of a species in the stands of a specific community. The stands were about five acres in area, and he classified the species in five classes: 90, 70, 50, 30 and 10%. This method and a coverage classification of four classes were used in an investigation of the vegetation of Kerr County, Texas, in relation to livestock and deer. The methods and characteristics used, "presence" and "cover", brought out very nicely differences in the composition of the vegetation of the different types and also the effects of overgrazing on the plant composition (22). The presence method was used to investigate prairie relics in Wisconsin over a period of more than ten years (67), in which stands of about four acres were used and a total of 65 in 45 townships in 15 counties. Only 19 of the total of 237 species in the 65 relics occurred in 50% or more of the stands, the most common being *Andropogon furcatus, Euphorbia corollata, Amorpha canescens, Solidago rigida* and *Coreopsis palmata.* All of these 19, except seven, had a presence of 50% or more in 20 stations in Iowa, Minnesota, Illinois, Kentucky and Ohio. This uniformity extended also to other species which occurred more rarely. The tendency of Wisconsin species to occur also in prairies in these other States is shown by the highly significant correlation between the lists of species, with a coefficient of 0.681. The term "index of homogeneity" was proposed to designate the percentage of species which occur in at least 50% of the stands ("constants"), expressed as a percentage of the total number of species in all of the stands in the particular vegetation type, such as low prairie. The index of homogeneity for the low prairie was 29.6%; high prairie, 21.8%; dry lime prairie, 26.2%; sand prairie, 10.5%; for the Wisconsin prairies as a whole (65 stations), 8%; and for the 20 stations in the five-state list, 8.6%. "Preferential" species were selected from the "constants" (presence of 50% or more), as those with a presence in one type at least 10% greater than in any other type. For example, "preferential" species, which also showed high fidelity, in the low prairie were *Spiraea alba, Equisetum arvense, Zizia aurea, Prenanthes racemosa* and *Thalictrum dasycarpum.*

DOMINANCE. This is the characteristic of vegetation which expresses the differential development of plants in a community so that some are thrifty and others are more or less suppressed in vigor or size. Usually the dominants are those species whose influence upon the habitat and whose reactions upon other species "are the most controlling both in kind and amount, and thus they (dominants) determine the conditions under which all the remaining species are associated with them" (46). Instead of regarding dominance as the extent of the surface covered or the volume occupied by plants (18, 24, 296), it appears more appropriate to consider dominance as one of the synthetic characteristics because all of the analytical characteristics play a more or less important part in determining the dominance of a species. *Andropogon furcatus* is often the most important dominant in the tall grass prairie, for many reasons, such as forming the uppermost layer of herbage, deep root system, early tillering, long growing season, high degree of vitality, densely sodded life-form which makes it a most effective competitor, high degree of sociability, large number of stalks per unit area, almost complete foliage cover, tallness, dry weight of 400 to 725 grams per sq. m. indicating vigorous growth and good adaptation to the environment where it grows, and wide distribution (289). Adamson (1) concluded that dominance on Table Mt., South Africa, is by life-forms rather than by species, and that this is one of the characteristics of a very old long-established flora. *Bromus tectorum,* an annual, has become dominant on large areas in western U. S. (252) since 1900, due to its large production of seed, rapid germination of seed and rapid growth of roots and shoots under only fairly favorable conditions, early maturity, adjustment of growth and maturation to unfavorable conditions, and large number of plants per unit area (average of 572 per sq. ft. in numerous counts in southern Utah).

SUMMARY

During the past decade or so increasing attention has been given to refinements in methods and procedures in the study of grassland communities and to more complete characterizations of communities. Advances have been made toward standardization, but it is premature at the present stage of development of grassland ecology to expect rigid formulations of research procedures. It is evident in recent publications that the trend is toward selection and further refinement of methods which will measure as objectively as possible the characteristics that are most significant in a particular investigation. For a complete analysis and description, all characteristics of the community require study.

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PHOTOTROPISM. II[1]

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INTRODUCTION

In the decade which has elapsed since the appearance of J. van Overbeek's review (53), relatively few investigators have concerned themselves directly with the question of phototropism. This is probably at least partially due to the feeling among plant physiologists that the phenomenon of phototropism is reasonably well understood in terms of auxin and differential growth. Despite this feeling, the present reviewer believes that some of the commonly accepted ideas involved in our understanding of phototropism may be subject to considerable reinterpretation. This paper will therefore be presented in two parts, a review of the new literature and a reconsideration of some of the accepted concepts of phototropism.

Before turning to the body of this paper it would seem desirable to summarize in a paragraph the facts and theories regarding phototropism as presented in the previous review. Phototropism is the curvature of a plant organ elicited by a unilateral light stimulus, and is generally brought about by a difference in growth rates on the illuminated and shaded sides of the organ. In the *Avena* coleoptile and a few other plant organs these different growth rates have been shown to be correlated with and presumably caused by different concentrations of auxin. Recognized ways in which light may influence auxin include inactivation, a directed migration towards the shaded side of the organ, and alteration of rate of production. Blue light is most effective phototropically and is presumably absorbed by a sensitizing pigment, generally assumed to be a carotinoid. It has been held that only auxin-*a* produces differential effects in light and dark, indoleacetic acid (IAA) acting the same under both conditions. The magnitude of the curvature response is dependent on the amount of light energy impinging on the tissue, irrespective of its distribution in time. In stems small amounts of energy generally produce positive curvatures (toward the light), and higher amounts may produce negative curvatures (away from the light). In the *Avena* coleoptile there are two separate growth reactions, one due to absorption of small amounts of light in the extreme tip, the other due to absorption of larger amounts of energy in the base. The pigment systems involved in these reactions are possibly different. The mechanism of phototropic bending in *Phycomyces* sporangiophores and other fungal organs can not be interpreted in terms of auxin, since no auxin requirement for growth of these organs has ever been demonstrated.

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[1] Supplement to article in The Botanical Review **5**: 655–681. 1939.

As will be made clear in the subsequent discussion, some of the statements in the paragraph above require extensive revision.

RECENT LITERATURE

LIGHT AND AUXIN. Stewart and Went (50) studied the light stability of the auxin in *Avena* coleoptiles. They found the ether-extractable auxin to be partially destroyed by light inside the coleoptile but stable to light after extraction. This difference in behavior in vivo and in vitro is presumably due to the presence of sensitizing pigments in the coleoptile. Light was found to be without effect on the diffusible auxin of the coleoptile inside or outside the coleoptile. Subsequently Went (57) was able to show that tropistic response of decapitated *Avena* coleoptile is correlated not with extractable but with diffusible auxin. Since Stewart and Went found no destruction of diffusible auxin by light, we must conclude that light acts by affecting not the total amount of auxin but only the relative amounts on the illuminated and darkened sides of the coleoptile.

Oppenoorth (39, 40) investigated the effect of various intensities and qualities of light on the ether-extractable auxin of dark and light halves of unilaterally illuminated *Avena* coleoptiles. He obtained evidence for photoinactivation of auxin in the regions of the first positive curvature and second positive curvature. With exposure to higher quantities of radiant energy, auxin synthesis was increased on the illuminated as opposed to the darkened side. Although he could obtain no convincing evidence for lateral transport of auxin in phototropism, Oppenoorth believes it to occur on the basis of experiments on geotropism and an experiment of Boysen-Jensen's which we shall discuss later. In this regard it is interesting to note that Burkholder and Johnston (10) failed to find evidence favoring lateral transport, although they too were able to demonstrate auxin photoinactivation.

Oppenoorth (40, p. 537) disagrees with the view expressed by Blaauw (4) that phototropism is merely a special case of growth inhibition by light, the so-called "light-growth reaction". He found that when auxin inactivation could be demonstrated, it occurred equally on the two sides of the coleoptile and therefore could not lead to differential growth. However, if one accepts the view of Went (57) that diffusible auxins and not extractable auxins are related to tropistic response, then Oppenoorth's evidence, based on extractable auxin, is not relevant.

Yamane (59) covered half of the horizontal intact leaves of *Fatsia japonica* with tinfoil and then exposed the leaves to 400–3900 lux[2]. In cases where the leaves showed positive phototropism, the ratio of auxin in the dark side to auxin in the light side was 1.53; when the leaves were negatively phototropic this ratio was 0.47, and when they were non-responsive the ratio was 0.97. Thus it would seem that auxin changes parallel and possibly are responsible for the movement of these leaves.

Kögl, Erxleben and Koningsberger (33) have reported that auxin-*a* is relatively light-stable, but that its lactone is easily inactivated by ultraviolet light. Although these workers previously (31) had found auxins *a* and *b* not to absorb ultraviolet light of wavelength longer than 2500 Å, they now (33) report an absorption peak at approximately 2900 Å, which they attribute to some unknown photochemical decomposition product of auxin-*a*-lactone. It is interesting that pure IAA has an ultraviolet absorption peak at about 2800 Å and an absorption spectrum almost identical with that of Kögl's hypothetical decomposition product of auxin-*a*-lactone. Another surprising finding is that approximately 3×10^6 molecules of auxin-*a*-lactone are inactivated by the absorption of one quantum of light. Although such high quantum yields could theoretically result from a light-initiated chain reaction, the value cited is so extraordinarily high for reactions in solution as to be absolutely unique. Perhaps the compound being photoinactivated is already in an unstable condition, so that the decomposition does not give an accurate picture of the true quantum yield.

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[2] One lux = one lumen incident per square meter, or one meter-candle. 10.76 lux = one foot-candle.

In corroboration of the work of Schuringa (45), Kögl and coworkers (30) found acid conditions to favor photoinactivation of auxin-*a*-lactone. Kögl and Verkaaik (35) noted about 96% of the auxin of *Phycomyces* sporangiophores to be destroyed by acid and to have a molecular weight (by the diffusion technique) of about 175. This is clearly IAA. The remaining 4% was sensitive to alkali and had a molecular weight of about 330, thus satisfying the requirements for auxin-*a*. Reference is also made to an unpublished experiment of Koningsberger and Verkaaik who applied *Phycomyces* auxin to twice decapitated *Avena* coleoptile and obtained regeneration of phototropic sensitivity and greater auxin destruction on the illuminated side. The auxin obtained from various fungi has a molecular weight (by diffusion) of about 175 and thus is presumably IAA. Notwithstanding this fact and because of their belief that indoleacetic acid can not be operative in light-growth reactions, Kögl and Verkaaik attribute this light growth response to destruction of auxin-*a* via its lactone. However, as will be evident from the subsequent discussion, IAA may also be involved in light growth reactions.

The evidence that only auxin-*a* may be involved in light growth responses was summarized in the previous review (53) on pages 667 and 676. However, Schneider (43) with *Avena* first internode sections, and Galston and Hand (26) with etiolated pea epicotyl sections have shown that light inhibition of growth (the "light-growth reaction") can also occur with IAA, α-naphthalene acetic acid and 2, 4-D as the auxin. Since IAA can participate in the "light-growth reaction", its participation in phototropic curvature is also possible.

Wildman and Bonner (58) have investigated the nature of the native auxin of *Avena* coleoptile. They were able to show that the enzyme which converts tryptophan to IAA is most concentrated in the tip of the coleoptile where auxin is known to be produced. The cold ether-extractable auxin is also almost entirely confined to the tip and was shown to be IAA by the Salkowski colorimetric technique. The diffusible auxin was collected and found to give a positive Salkowski reaction and to resemble IAA in its diffusion constant, provided that it is first subjected to ether extraction. The authors conclude that the bulk, if not all, of the native auxin of *Avena* coleoptile is IAA. This would also imply that IAA must be involved in the light-growth reactions of *Avena* coleoptile.

PIGMENTS. Kögl and Schuringa (34) have reported that auxin-*a*-lactone, which is normally inactivated only by ultraviolet light, may be inactivated by visible light in vitro, provided one of several carotinoids is present as a sensitizing pigment. They regard this as the system operative in vivo, based also on their belief that auxin-*a* is the native auxin of higher plants.

Galston (23) has shown that IAA may be photoöxidized by blue light in vitro in the presence of riboflavin as a sensitizer. The action spectrum for this reaction (25) follows closely the absorption spectrum of the pigment. If riboflavin is added to the medium in which etiolated pea epicotyl sections are being grown, then severe growth inhibition occurs in the light but not in the dark. If IAA is mixed with a brei of etiolated pea epicotyls, the action spectrum for photodestruction of the IAA is almost identical with that of the IAA-riboflavin reaction. This definitely establishes riboflavin as a major receptor pigment in the destruction of auxin by plant tissue. The quantum yield for the IAA-riboflavin reaction is approximately 0.7 molecule of IAA disappearing per quantum of blue light absorbed.

Riboflavin is abundant throughout the *Avena* coleoptile, occurring uniformly to the extent of about 30 γ per gram dry weight (25). The diffusible auxin is fairly light-stable outside the coleoptile, but when incorporated into an agar block containing 2 γ per cc. of riboflavin, it is rapidly photoinactivated. The rate of inactivation is similar to the rate of photoinactivation of pure IAA under identical conditions. This is taken as evidence that riboflavin could be the sensitizing pigment and IAA the auxin involved in the phototropic response. The action spectrum for the first positive curvature of *Avena* coleoptiles has some points of resemblance with the absorption spectra of both β-carotene and riboflavin, being in general closer to the carotene curve. It is, however, considered by Galston and Baker (25) that in vivo action spectra are not sufficiently precise to distinguish between two pigvalue cited is so extraordinarily high for reactions in solution as to be absolutely unique. Perhaps the compound being photoinactivated is already in an unstable condition, so that the decomposition does not give an accurate picture of the true quantum yield.

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Oppenoorth (40), although he subscribes to the view that a carotinoid is the photoreceptor in phototropism, obtained a total pigment extract of *Avena* coleoptiles which showed a single absorption peak at about 4450 Å (p. 310). This is precisely the absorption peak of riboflavin, and the data should therefore not be used as evidence in favor of the carotinoid theory. Bünning (9), in the third paper of his series on phototropism and carotinoids, obtains a single-peaked action spectrum for phototropism in *Pilobolus* and *Phycomyces,* as had Castle (12) several years before. He explains the lack of a double peak (found in the absorption spectrum of β-carotene) in terms of differential absorption of different wavelengths on the near and far sides of the coleoptile, assuming that at $\lambda = 4500$ Å 10% of the light is absorbed on the near side. It would seem equally logical to this reviewer to interpret the single peak as real and as due to absorption by flavin. Since riboflavin is known to sensitize the photoinactivation of tryptophan, histidine, methionine and enzymes as well as of IAA (23, 24), the curvature of these fungal sporangiophores need not be interpreted in terms of auxin. Bünning also shows that the distribution of carotene crystals parallels sensitivity to light in the *Avena* coleoptile, although the pigment is deficient in the extreme tip, which is most sensitive to light. The existence of a gradient for carotene and the lack of such a gradient for riboflavin may be considered as support for the carotene theory.

Bünning confirms a previous report by Atkins (1) that green plants respond phototropically to red light. Atkins had found this for green *Avena* seedlings as well as for green plants of *Lepidium* and *Celosia;* Bünning confirms it with *Sinapis alba* and *Helianthus annuus.* It it interesting to note that this phenomenon had previously been reported by Zollikofer (60) and by Bachmann and Bergann (2) but had not received sufficient attention. Flint (21) finds red light ineffective on *Pilobolus* and Italian rye grass, the former in opposition to the results of Parr (41). If it is true that red light produces phototropic curvature, then some pigment other than carotene or riboflavin must be involved. Chlorophyll is a possible receptor in this region, as are various other tetrapyrroles. Manten (37*b*) has noted the phototropic response of the blue-green alga *Tolypothrix* to blue light to be enhanced by orange light which by itself is ineffective phototropically. He concludes that β-carotene is the photo-receptor for phototropism and that the effect of orange light is mediated through photosynthesis. This explanation might also apply to the observations cited above in which red light was found to effect phototropic curvature.

EFFECTS OF LIGHT ON THE PHYSICAL APPARATUS OF THE CELL. Bottelier (6) demonstrated that four to eight minutes after illumination of an *Avena* coleoptile, the rate of protoplasmic streaming was depressed, the magnitude of the effect being related to the intensity of the light employed. As with phototropism, blue is the most effective wavelength. If the wavelength employed was 4360 Å, the maximal inhibition of streaming was produced by 200 ergs/cm.^{2}, which produces a strong first positive curvature. If the intensity is raised to 800 ergs/cm.^{2}, streaming is actually accelerated. Bottelier suggests that this effect may be important in phototropism in that a decrease in the rate of streaming would decrease the transport of auxin from the tip to the growing cells. Since growth of the cells is limited by auxin, this light interference with auxin transport would lead to differential growth on the two sides of the coleoptile, accounting for the curvature towards the light. Unfortunately it is not entirely clear that auxin transport occurs as a result of protoplasmic streaming, so that the major assumption of Bottelier is still unproved.

Gessner (27) found that unilaterally illuminated *Helianthus* hypocotyls showed a decreased plastic extensibility on the lighted side. *Lupinus luteus*, which shows no phototropic curvature, does not display this effect. This author concludes that the effect of light on the cell wall properties may account for observed phototropic curvature. Bünning reports a similar observation for *Pilobolus* curving towards light. Brauner (8) found that blue light caused a great decrease in the turgor of the pulvinal cells of *Phaseolus multiflorus* and thought that the effect might account for the curvature. Red light was also effective, causing him to conjecture that chlorophylls, as well as carotinoids, are involved as photoreceptors.

With regard to electrical effects, Clark (15, 16) found no easily detectable relation between electrical polarity and auxin transport. For instance, the electrical polarity of a segment of coleoptile can be altered by gravity or light, but the polarity of auxin transport remains unaltered. In contrast with these results, Schrank (44–47) finds a direct relationship between electrical and curvature responses of the *Avena* coleoptile. The side of the coleoptile stimulated by light becomes electrically negative. Since auxin migrates as the anion, this polarization should presumably result in diversion of auxin to the shaded side, with a resulting curvature towards the light. Application of 10 μ amperes of current anywhere on the coleoptile results in a transverse polarization and bending. The curvature is at first towards the positive side, later towards the negative side.

Using 10 MCS of light and 5 μ amperes of current, each of which produces a half-maximal curvature when used alone, Schrank found current to antagonize the light effect when the positive pole is on the shaded side and to augment the light effect when the positive pole is on the illuminated side. This presumably establishes a common pathway and common limiting factor to the two processes. One may conclude, therefore, that changes in bioelectric potentials are associated with phototropic curvature, perhaps in a causal connection. Du Buy and Olson (19) have analyzed the data of Clark (15, 16) and have concluded that instead of showing no correlation between transport and applied current, they show that these two factors are related. This would bring Clark's experiment into agreement with those of Schrank. In view of the convincing nature of Schrank's recent experiments, such an interpretation would seem reasonable.

Wassink and Bouman (55) have analyzed the original data of Blaauw in an attempt to determine how many quanta of light are essential for initiation of phototropic bending in *Phycomyces* and in *Avena*. They assume that if K quanta are required for a reaction to occur, then the chance of the reaction occurring is proportional to the average quantum content of the light flash, according to Poisson's formula. Therefore, if the per cent of curving *Phycomyces* sporangiophores is plotted against the log of the average number of incident quanta, an S-shaped curve results.

The steepness of slope of this curve is a function of the number of quanta required for the reaction to occur.

The plot for *Phycomyces* indicates that absorption of one quantum may initiate bending; for *Avena* the figure is much higher. If this analysis is correct, it would imply that in *Phycomyces* a "chain reaction" is initiated by absorption of the quantum. This chain reaction might involve the auxin photoinactivation described by Kögl and co-workers (33), in which 3×10^6 molecules of auxin are destroyed per absorbed quantum. The main difficulty with such a hypothesis lies in the fact that auxin is not known to be essential for the growth of *Phycomyces*.

Van Overbeek (53, p. 662) states that the Weber-Fechner law holds for phototropism in only a few special cases. However, Pringsheim (42) finds it to hold quite well for the reaction threshold, and Fuller and Thuente (22) find both the Bunsen-Roscoe and Weber-Fechner laws to be followed by all plants tested. Filzer (20) determined the minimal light causing curvature, using both unilateral and bilateral illumination. In the latter situation 17 times as much light is required for curvature. This fact is of interest in view of the widespread use of opposing lights in determining action spectra for phototropism. Burkholder (10) found phototropic curvature to be independent of the angle of incidence of the light.

PHOTOTROPISM IN ROOTS. Naundorf (38) showed that the primary roots of *Helianthus annuus* are negatively phototropic, curving up to 40°. The tip is the most perceptive region, decapitated roots not responding. Replacement of the tip by agar containing IAA gave no curvature, indicating that the tip has other functions in the curvature besides that of auxin supply. These other functions are presumably related to light perception.

Excised roots were also found to respond, although their curvature was less than that of intact roots. Root tips produce auxin, more being produced in the light than in the dark. Growth is also greater in illuminated than in darkened cultures. In a unilaterally illuminated root, the light side produces more auxin than the dark side. Blue light is most effective in producing curvature and in increasing the auxin content of the illuminated side. Segelitz (49) has also reported that light increases the auxin content of isolated roots.

From these data it is clear that phototropism in these roots involves the auxin production mechanism. Since roots are known to be inhibited by very low concentrations of exogenously applied auxins, it would be logical to expect a positive curvature if light increases auxin synthesis, as it apparently does. The fact that negative curvatures result from the higher auxin levels in light indicates that the endogenous auxin level is sufficiently low that auxin is still limiting to growth. Thus a light-induced increase in auxin synthesis results in a growth acceleration on the near side and a negative curvature.

Dassek (17) finds the rhizoids of various liverworts to be phototropically responsive, the direction of the response to light depending on the refractive index of the medium in which they are exposed. Thus they are negatively phototropic in air or water, positively phototropic in paraffin oil. This effect had previously been obtained and satisfactorily explained by Castle (13).

MISCELLANEOUS OBSERVATIONS. De Ropp (18) noticed that soaking the grain before excision of the embryo increases growth and tropistic response of Petkus winter rye. This is presumably not an effect on the phototropic mechanism itself but rather on growth, probably by way of supplying growth stimulatory substances from the endosperm.

Langham (37) attributed the erect or prostrate habits of certain plants to their positive phototropism at low light intensities and negative phototropism at high light intensities. Experimentally the behavior of his plants seemed to support this hypothesis, since they were erect in darkness and prostrate in bright light.

Chakrabarty (14) reported that the sporophore of *Agaricus polyporus* is positively phototropic during its period of active growth and negatively phototropic after formation of the apical knobs. The reason for this change-over is unknown.

A REEXAMINATION OF SOME ACCEPTED CONCEPTS OF PHOTOTROPISM

IS CAROTENE THE PHOTORECEPTOR[3]? The evidence leading to the view that carotene is the receptor pigment in phototropism is threefold:

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[3] Bandurski and Galston (unpublished) have recently found that the carotinoidless coleoptiles of an albino mutant of *Zea* are able to respond to a phototropic stimulus almost as well as the wild-type strain from which they were derived. Since the mutant contains the normal amount of riboflavin, this experiment is strong support for the view that riboflavin, not carotene, is the photoreceptor for phototropism.

a). Carotenes may be demonstrated to occur in the receptor regions of phototropically sensitive organs (9).

b). Carotenes may sensitize the photoinactivation of auxin-*a* in vitro (34).

c). The "action spectrum" for the phototropic response corresponds reasonably well with the absorption spectrum for a carotinoid (30).

This would be convincing evidence were it not also true that the same three statements may be made about another yellow pigment, riboflavin. This pigment occurs in abundance in the coleoptile (25), will sensitize the photodestruction of IAA (23), and has an absorption spectrum which corresponds well with several action-spectra for phototropism (25). In general it may be said that action spectra for the phototropic curvature of *Pilobolus* and *Phycomyces* (9, 12), and that for the "base reaction" of *Avena* coleoptile (29), are closer to the absorption spectrum for riboflavin than to that of a carotinoid. However, the action spectrum for the "tip reaction" (25, 30) seems more closely related to a carotinoid than to riboflavin.

The question then arises: How accurate is an action spectrum in the determination of the photoreceptor pigment? Blum (5, pp. 56–60) discusses this matter in some detail:

"Assuming the system to obey Beer's law at all concentrations, it should be possible to calculate the absorption of any given thickness of solution from the absorption or extinction coefficient. However, it is usually impossible to know the concentration, let alone the thickness of the material after it has been introduced into a given biological system where it is to act as a photosensitizer, so it would seem impossible to make a fair comparison of the absorption spectrum of a dye with the action spectrum for a biological system sensitized by that dye".

When one adds to this difficulty the possible complicating effects of pH, screening pigments and adsorption-orientation effects on light absorption by the pigment in vivo, it should be clear than an action spectrum is not sufficiently precise to distinguish between two pigments whose absorption spectra are as similar as are those of riboflavin and β-carotene. Even the criterion of one peak vs. two peaks is not entirely valid, since a flavin-flavoprotein mixture may have a two-peaked absorption curve (25), and a carotinoid may yield a one-peak action spectrum (9).

Loofbourow (37*a*), discussing the significance of action spectra, concludes:

"In view of these many assumptions, and of further difficulties in the biological aspects of the experiments, it may seem surprising that action spectra have yielded any useful information. Fortunately, if one is aware of the perturbing factors, one can hold them within reasonable limits in many instances, so that useful interpretations of the data are possible. . . . Probably the chief pitfall to be avoided is the assumption, apparently not uncommon, that because the method involves careful measurements with elaborate physical equipment it must necessarily yield meaningful information".

The lack of interest in riboflavin as a receptor pigment for phototropism is probably an accident of chronology. The first precise action spectra for phototropism (30) were obtained at a time when riboflavin had just been discovered and was not generally available for experimentation. Thus early suggestions regarding the similarity between action spectra for phototropism and absorption spectra of carotinoids deflected the attention of subsequent investigators from other possible photoreceptor pigments. Had the flavins been generally known prior to 1934, they might very well have received greater consideration in this connection.

DOES LATERAL TRANSPORT OF AUXIN OCCUR UNDER THE INFLUENCE OF LIGHT? The most definitive evidence for light-induced lateral transport of auxin came from the experiments of Went (56). He showed that if the diffusible auxin content of an unilluminated *Avena* coleoptile tip be regarded as 100, then in the first half hour after illumination it is only 72, in the second half hour it is 88, and in the third half hour it is 87. If a tip is unilaterally illuminated with 1000 MCS of light, the diffusible auxin released by the illuminated half in about 84 minutes is 27, and from the shaded half 57. (The control half is here taken as 50.) By a subsequent experiment in which tips were unilaterally exposed to 100 MCS of light he obtained the following results:

Time after illumination	Curvature in degrees produced by diffusible auxin		
	Light side	Dark side	Sum
1st 75 min.	6.8 ± 0.4	9.6 ± 0.8	16.4
2nd 75 min.	1.8 ± 0.8	15.0 ± 1.0	16.8

Because the distribution of auxin between light and dark sides was altered without any significant change in the total diffusible auxin present, Went concluded that there had been a lateral migration of the auxin.

Another interpretation of these data is possible. It has been shown by van Overbeek (54) that much more diffusible auxin is released from coleoptile tips over an extended period of time than can be extracted at any one time. This implies that the release of diffusible auxin is the result of its continuing synthesis in the tip. It is therefore possible to hypothecate the following mechanism: (*a*) the total quantity of auxin produced by the tip is limited by the amount of auxin precursor coming up the coleoptile; (*b*) the enzymatic auxin-producing apparatus on the "light" side is partially inactivated by light; (*c*) less of the auxin precursor which appears at the tip will then be converted to auxin on the illuminated side; (*d*) the unconverted precursor will then randomly diffuse to the "dark" side, where it will be converted to auxin. Such a mechanism would account for constancy of total auxin production, together with difference in distribution between illuminated and darkened sides, without invoking a light-induced transverse migration of auxin. The fact that riboflavin (and presumably other pigments) is known to sensitize the photoinactivation of various enzymes (24) would lend credence to this view.

The data of van Overbeek (51, 52) have also been conventionally interpreted in terms of a light-induced transverse auxin migration. Using *Raphanus* seedlings, this investigator was first able to show that symmetrical illumination decreased the growth response to auxin but did not influence the quantity of auxin transported through hypocotyl cylinders. Unilateral illumination of the tips caused less auxin to diffuse from the lighted than from the shaded side. The same was true if the tip were removed and replaced by an auxin-containing agar block. Finally, if an auxin-containing agar block were placed asymmetrically over the lighted side, more auxin could still be obtained by diffusion from the darkened half. From these experiments it is quite clear that lateral migration of auxin may occur. Yet the question remains: Is the lateral migration caused by the light? It is certainly equally possible to account for all these data on the basis that auxin moves laterally, uninfluenced by light, but that differential photo-inactivation occurs on the illuminated and shaded sides. This obscure point could be resolved by an experiment in which balance sheets for auxin in darkened and illuminated tissues are carefully compared.

It is not the purpose of this reviewer to press for the acceptance of this alternative view, but merely to point out that a light-induced lateral transport of auxin has not yet been unequivocally demonstrated. The best evidence for stimulus-induced lateral transport of auxin comes from experiments on geotropism, where the phenomenon seems reasonably well established. The single most convincing experiment in the field of phototropism is probably that of Boysen-Jensen (7) who found that insertion of a physical barrier in the middle of the *Avena* tip prevented phototropic curvature if the barrier was perpendicular to the direction of the light beam, but not if it was parallel to it. The present reviewer has been unable to confirm this experiment (unpublished), but even confirmation would not prove that it is auxin and not precursor which is moving laterally.

Also worthy of note is the experiment of Laibach (36) who found that auxin applied subsequent to illumination increased the curvature due to light. Since this obviously could not be due to photoinactivation of the added auxin, he suggested that the auxin had migrated to the shaded side under the influence of a light-induced polarity. If this is the true explanation, then possibly the effect is caused by the light-induced decrease in cytoplasmic streaming on the near side (6) with a subsequent lateral transfer due to local accumulation of the auxin. We must conclude that, although certain experiments suggest a light-induced transverse migration of auxin, this point has by no means been proved.

CAN LIGHT-GROWTH RESPONSES BE INTERPRETED ONLY IN TERMS OF AUXIN-A? Van Overbeek (52) conducted experiments which led him to the conclusion that if auxin-*a* is supplied to tissue, light will decrease its effectiveness ("light growth reaction"), but that if IAA is supplied to the tissue, light has no effect on its action. The *Avena* experiment was conducted as follows. Coleoptiles were twice decapitated, and then an agar block placed on the top asymmetrically, as in the *Avena* test. One set of plants was illuminated symmetrically and another set kept in the dark room. If IAA was the auxin in the block, no difference in curvature occurred as a result of the light treatment, but if auxin-*a* was used, light decreased the resultant curvature. Van Overbeek mentions, however, that since no pure auxin-*a* was available, he was forced to utilize coarse corn meal as his source of this product. He merely soaked a plain agar block in an aqueous corn meal extract for a few hours and then used it as his source of auxin-*a*, assuming this to be the auxin present because the work of Kögl, Erxleben and Haagen-Smit (32) had shown auxin-*a* to be present in corn oil. Yet we now know that corn endosperm contains large quantities of IAA (3) and also adequate quantities of sensitizing pigment, such as riboflavin and carotinoids. In view of the known flavin-sensitized photoöxidation of IAA (23) it is possible to interpret van Overbeek's results solely in terms of pigment-sensitized photo-inactivation of IAA in the agar block. The section growth experiments of Schneider (43) and of Galston and Hand (26) further reinforce the view that light-growth responses may be obtained with IAA as the auxin.

It must be mentioned that van Overbeek obtained similar results with pure auxin-*a* from Kögl's laboratory, as did Koningsberger and Verkaaik (35*a*). If this product was actually devoid of any trace of photosensitizer, the result supports van Overbeek's thesis. Obviously this crucial experiment needs to be repeated, and it is hoped that pure auxin-*a* will once again become available for experimentation so that a definite decision on this question may be made.

SUMMARY

The major revisions which we must make in our ideas concerning phototropism are the following:

a) Perception of blue light by phototropically reactive plant parts may be mediated by one or more of several pigments, all of whose absorption spectra agree reasonably well with the action spectrum for phototropism. These pigments include various carotinoids and riboflavin-containing compounds.

b) Lateral transport of auxin under the influence of light, long accepted as a mechanism explaining the auxin changes which accompany phototropic bending, has not been unequivocally demonstrated. The data could equally well be interpreted in terms of a disturbance of a substrate-limited auxin-producing mechanism at the apex of the coleoptile and other plant organs.

c) Indoleacetic acid can participate in light-growth reactions by virtue of its rapid photoinactivation in the presence of the proper sensitizing pigments.

d) Photoinactivation of auxin may be readily demonstrated in vitro and in vivo, but the relation of auxin photoinactivation to phototropism is still not clear. The effect of light on auxin levels of tissues may be mediated not only by a photoinactivation mechanism but also by a stimulation or inhibition of the auxin-producing process, as well as through effects on auxin migration.

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THE FLOWER CONSTANCY OF BEES

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INTRODUCTION

The flower constancy of the honey-bee was first accurately described by Aristotle (2) in the following terms: "On each expedition the bee does not fly from a flower of one kind to a flower of another, but flies from one violet, say, to another violet, and never meddles with another flower until it has got back to the hive". The phenomenon was well known to the 19th century floral biologists, Charles Darwin, Herman Müller and their colleagues (5, 6, 18, 21, 37, 53, 54). It was subsequently investigated by several students of insect behavior at the turn of the century, particularly by Felix Plateau, Gaston Bonnier, August Forel and Karl von Frisch (9, 26, 27, 29, 61). More recently the bearing of bee constancy on certain practical aspects of agriculture, such as problems of orchard management (10, 11, 13, 15, 16, 51), production of forage crops (40) and contamination of seed crops (4, 20), has come to the fore.

The flower-visiting behavior of the honey-bee, which is a domesticated animal, has thus been the subject of numerous studies during this century and the last. One of the purposes of the following review is to summarize this scattered information for botanists. The flower-visiting habits of wild bees are not so well known as those of the honey-bee. An attempt has nevertheless been made to summarize the principal published observations on flower constancy in wild bees. These observations indicate that wild bees possess an instinct of flower constancy similar in general to that of honey-bees. The writer will undertake, finally, to consider some of the possible consequences of the phenomenon of flower constancy in the evolution of the angiosperms.

THE PHENOMENON OF FLOWER CONSTANCY

A division of labor exists among field bees of *Apis mellifica* in that certain workers carry water, others gather pollen, nectar or propolis (resin from bark and buds), and still others search for new sources of food (9). The instinct of a given individual to remain constant to a special occupation is quite rigid, moreover, and is maintained over a period of days (57). Only pollen- or nectar-gathering bees, therefore, will have an opportunity to manifest the phenomenon of flower constancy, and in the discussion which follows we shall be concerned with the flower-visiting workers alone.

OBSERVATIONS OF FIELD BEES. The contention that pollen-gathering honey-bees remain true to one species of plant at a time has been confirmed both by direct observation of bees in the field and by examination of their pollen loads when they return to the hive. The observational evidence accumulated by two English naturalists of the 19th century (6, 18) with respect to the honey-bee and the bumble-bee is summarized in Table 1. The observations of Plateau (61), which were not quantitative, brought that author to the same conclusion, namely, that species of bumble-bees are relatively inconstant, whereas the honey-bee is highly constant, though even she occasionally strays from one kind of flower to another.

TABLE 1

PROPORTION OF INTRASPECIFIC TO INTERSPECIFIC VISITS MADE BY HONEYBEES AND BUMBLE-BEES, FROM OBSERVATIONS OF BENNETT AND CHRISTY IN 1883

Genus	Authority	Intraspecific visits, per cent	Interspecific visits, per cent	Number of individuals observed	Number of flowers visited
Apis . .	(18)	100.0	0.0	8	258
	(6)	83.3	16.7	6	+ 45
Bombus	(18)	51.0	49.0	51	+1,660
	(6)	69.7	30.3	33	+ 417

Only gradually, by the use of marked bees and other improvements of technique, are we coming to an appreciation of all that is subsumed by this word "constancy". Hermann Müller (53) was the first to discover, by following honey-bees daubed on the thorax with colored paints, that constancy is maintained not only throughout a given foraging flight but also over a period of days. He thus observed one marked bee to return repeatedly during 11 consecutive days, and another during ten days, to the same plant of *Salvia aethiopis* in his garden.

Minderhoud (51) subsequently demonstrated, from observations of marked honey-bees on local patches of *Taraxacum officinale, Trifolium repens* and *Reseda odorata,* that, given an abundant supply of flowers of one species, the foraging bee instinctively visits an area about ten meters square. Butler, Jeffree and Kalmus (16) place the diameter of the fixation area at about five yards for patches of *Epilobium angustifolium* and *Echinops sphaerocephalus.* One case has been reported where individual honey-bees returned day after day to one individual plant of a group of three bushes of *Cotoneaster* (17). These observations suggest that the constancy of honey-bees to one kind of flower may be related to their constancy to one locality.

In view of the known heterozygosity of many cultivated plants which are pollinated almost exclusively by honey-bees, the foregoing results on local constancy would appear to explain too much. Butler and his co-workers (13, 15, 16) have, however, extended and clarified the observations of Minderhoud. The situation as described by Butler seems to be as follows. The first flowers to bloom in the spring will have a group of bees fixed on them, and it is at this time that territorial repartition is instituted. Bees from other hives, arriving on the scene after the available local patches have all been pre-empted, will not at first be able to forage as a stable group in the manner of their predecessors, but will wander over the field more or less at random. These wandering bees are responsible for much of the wide cross-pollination within the plant population.

But as a second species of plant comes into flower, it is occupied by the erstwhile wanderers which now likewise become constant. This second source of pasturage, even if it is more abundant than the first crop, does not attract the older bees, and, of course, newcomers will now as before have to serve their term as wanderers before they too can find the means to a constant source of pollen.

As a species of plant approaches the end of its blooming period and the number of flowers dwindles, a brief period of competition for pollen ensues, leading to a new wave of inconstancy. The equilibrium between the bees and their pasture is soon reestablished, however, by the natural death of the older bees. The dwindling crop of pollen attracts no young foragers from the hive; forewarned by a communication from the older workers (30, 56), they turn instead to new and more fertile fields.

Butler, Jeffree and Kalmus (16) rightly note that the local constancy of bees is not without important consequences for the genetics of plant populations. Bee plants, even though they occur in a large continuous population and are self-incompatible, "will still not form a homogeneous interbreeding population but rather an aggregate of small visiting systems in the sense of Wright (1921) which do not exceed a few yards in diameter. In short the local constancy of bees results in a considerable degree of inbreeding in the pollinated plants". It is quite possible, therefore (and the point deserves further attention), that both the heterozygosity of bee plants and the panmixia of their populations are less than has commonly been supposed.

The experience of the British seed trade tends to confirm this view. That trade, faced during World War II with the problem of maintaining uncontaminated the pedigrees of different vegetable plants—turnips, radishes, and the like—while producing them in mass on a crowded island, turned to an experimental study of intervarietal crossing by bees (3). Crane and Mather (20) concluded that if a large mass of flowers were available, bees would confine their visits to a small area. When radishes are grown in quantity, for example, an interval of 300 feet guards against contamination by intervarietal crossing. The 300 feet in this case is a conservative isolation distance. Bateman (3) found that the first 50 feet reduced the contamination to 1%; the next 200 feet in Crane and Mather's study diminished it further only to 0.1%, even in the vicinity of a bee-hive.

The conclusion that the local constancy of honey-bees leads to a restriction of panmixia in many plant populations has also received some unexpected support from the field of plant pathology. The fire-blight pathogen, a bacterium (*Erwinia amylovora*), is transmitted by bees from blossom to blossom of apple and pear. The pathogen thrives best in nectar of low concentration; it does not do well in nectar of high or medium concentration (33). "However, in many cases in which the nectar was at a favorable concentration, little or no infection occurred after contaminated bees had sipped from it. It is, therefore, apparent that other factors besides nectar concentration are important in limiting blossom-blight transmission by bees" (33).

EXAMINATION OF POLLEN LOADS. Some data on the relative frequency of pure and mixed pollen loads in honey-bees and various wild bees are summarized in Table 2. The data of Brittain and Newton (10) were obtained in Nova Scotia and Quebec, mainly from bees on deciduous fruits; those of Clements and Long (19) were obtained at Pike's Peak, Colorado, from bees on wild plants; and the data of Betts (7) were assembled in England. It was convenient to summarize these data in Table 2 by genus, rather than by species, of bee. It is necessary to remark, therefore, that several of the genera, *Andrena* and *Halictus* for example, consist of numerous species which differ widely in flower constancy, so that a comparison of such large genera with the single species *Apis mellifica* would be quite unrealistic.

TABLE 2

PROPORTION OF PURE POLLEN LOADS TO MIXED LOADS IN SEVERAL GENERA OF BEES, FROM MICROSCOPICAL EXAMINATION OF THE PELLETS[1]

Genus	Authority	Pure loads, per cent	Mixed loads, per cent	Number of individuals examined
Andrena	(10)	43.5	56.5	180
	(19)	64.3	35.7	28
Apis	(10)	62.0	38.0	274
	(19)	87.5	12.5	32
	(7)	93.2	6.8	3,437
Anthophora...	(19)	20.0	80.0	5
Bombus	(10)	58.8	41.2	85
	(19)	49.0	51.0	102
	(7)	57.1	42.9	14
Halictus	(10)	83.9	16.1	247
	(19)	75.0	25.0	8
Megachile ...	(10)	75.0	25.0	4
	(19)	54.5	45.5	22

[1] Minor discrepancies exist at several points between the figures presented here and those given by the authors in their summaries; however, the above figures were compiled from the published data of the authors cited.

Considering the great variation in species of bees, species of plants, place and time, however, the results are on the whole fairly consistent. Brittain and Newton concluded that the honey-bee is not so constant as most authors have assumed; Betts, and Clements and Long, on the other hand, decided that she maintains a high degree of flower constancy. The species of wild bees belonging to the genus *Halictus* are the most consistently flower-constant for all sets of data.

The wild bee *Anthophora simillima* makes a poor showing in the sparse data of Clements and Long. It may be worthwhile, therefore, to mention the findings of Linsley and MacSwain with a different species, *A. linsleyi* (39). These investigators, combining field observations with an examination of both pollen loads and cell series, found that *Anthophora linsleyi* in two localities adjacent to the Mojave Desert, California, was collecting pollen only from *Salvia carduacea.* The bees by-passed other plants in flower to obtain the sage pollen, even though this made it necessary for them to fly more than a mile from their nest. From an examination of the cells it was evident that the bees provisioned each cell with a single type of pollen, usually the bright red pollen of *Salvia carduacea;* if a cell was found stocked with more than one kind of pollen, the different types were present in different parts of the cell. Bennett (5) long ago observed that in a field where *Lamium album* and *L. purpureum* were flowering together, *Anthophora retusa* visited only the former species of plant. Since the pollen of that species is yellow and that of *L. purpureum* is red, it was comparatively easy for Bennett to verify his observations by a microscopic examination of the pollen loads.

One may easily obtain an exaggerated notion, from the percentages of mixed loads in Table 2, regarding the frequency of interspecific visits by the bees. A pellet composed, let us say, 99% of pollen from *Geranium caespitosum* and 1% of pollen from *Ranunculus acicularis* and *Potentilla gracilis* does not, statistically speaking, point to a very high degree of inconstancy. Such cases are not rare. From Clements and Long's data it is possible to pick them out. Reanalysis of their data from the standpoint of relative degree of inconstancy leads to the following picture. Of the mixed loads of *Apis mellifica* represented in Table 2, every one was 95–99% pure. Most of the mixed loads of species of *Andrena* were similarly more than 95% pure, but two individual bees carried loads which contained more than 5% of foreign pollen. Over half of the interspecific visits paid by species of bumble-bees resulted in pollen pellets which were at least 95% pure.

Having made these allowances, however, we remain with the fact that a certain amount of flower inconstancy, sometimes more, sometimes less, is a normal ingredient of bee behavior. Why this is so is clear from Butler's account of the inevitable temporary disproportions between the numbers of individuals of bees and the extent of their pasturage.

THE PSYCHOLOGICAL BASIS OF FLOWER CONSTANCY

Sir John Lubbock's experiments with colored papers provided with drops of honey first demonstrated the existence of a color-sense in bees (47). These early experiments, however, were subsequently swamped out by Plateau's extensive writings. Plateau believed that insects are attracted to flowers neither by color nor by shape, but by odor (59; see also 19 for a complete review of Plateau). After a good deal of printer's ink had been expended on this point, Frisch performed in 1914 a series of experiments that were as crucial as they were clearly conceived (27).

Frisch's method was to arrange a set of small colored papers at random on a table; intermixed with the colored papers were several gray papers, forming a graded series from light to dark gray. The block of gray and colored papers was kept under a piece of plate glass in order to eliminate any possible factor of odor. Small watch glasses were next placed on the plate glass above each of the papers. One of the watch glasses, for example the one on the blue paper, was then filled with sugar solution. Honey-bees from a nearby hive were permitted to come to the table and soon found the syrup in this one watch glass. As often as the bees exhausted the supply of syrup in this watch glass, Frisch replenished it. While the bees were away at the hive, moreover, he frequently changed the arrangement of the colored and gray papers in the block, but always kept the supply of syrup in association with the color blue.

After the bees had been conditioned during two days to come to the blue paper for the syrup in the watch glass upon it, Frisch suddenly rearranged the order of the papers and provided syrup in all of the watch glasses. The bees nevertheless returned directly to the blue paper in its new position and ignored the other watch glasses. Or if all of the watch glasses were removed the bees still hovered over the blue paper, but not over any of the gray ones. Since the bees readily distinguished between blue and every shade of gray placed alongside it, they were not color-blind. Frisch asserted: "Die Biene besitzt Farbensinn!".

On the basis of numerous experiments of this type, Frisch (27) concluded that the honey-bee does not perceive red as a color but rather as black. In the remaining portions of the spectrum the bee has no fine sense of shades; she confuses every shade of blue, and blue with purple, and she fails to discriminate between green, yellow and orange. The bee, according to Frisch, thus sees only two main colors, blue and yellow, in addition to white and black.

Frisch notes in this connection that multicolored flowers in the European flora consist very largely of combinations of blue and yellow, blue and white, or yellow and white. Frisch also brings up the well known rule that flowers possessing the most elaborate contrivances for cross-pollination by bees are also predominantly blue- or purple-colored, a generalization which is usually "explained" by reference to a Lieblingsfarbe. The bee has no color preferences, according to Frisch, and if blue and purple predominate in bee flowers, it is probably because this color (singular) is precisely the one which, to the bee's eye, contrasts most strongly with the green of the foliage.

It is now known that honey-bees, as well as many other flower-visiting insects (the social bees *Bombus bimaculatus* and *Trigona cressoni parastigma,* the solitary bees *Xenoglossa pruinosa* (on *Cucurbita*) and *Melissodes bimaculata* (on *Convolvulus*), the wasp *Polistes annularis,* and some syrphid-flies), can see ultra-violet light (38, 38*b*, 48, 49). The tropical bee *Trigona cressoni parastigma* was thus able to distinguish between ordinary white cards and white cards that reflected ultra-violet in experiments carried out by Lutz (49). It is significant to note, therefore, that some flowers also reflect ultra-violet, for example, species of *Cucurbita, Lilium* and *Primula* (49). These facts suggest that discussions of the relationship between the colors of flowers and the behavior of flower-visiting insects must take into consideration the ultra-violet colors of many flowers.

The color-sense of bees consists, in summary, of the perception of four main color groups: *a*) a yellow group which includes vermillion, orange, yellow and yellow-green; *b*) blue-green; *c*) a blue group which includes blue, purple and violet; and *d*) ultra-violet (38*b*). Bees are color-blind for pure red (27, 38, 38*b*). Though they can distinguish different degrees of brightness as white, gray and black, they do not distinguish different shades within one color group. In these respects bees are similar to most other arthropods that have been investigated (38*a*).

The color-sense of the honey-bee is supplemented by a fine sense of form. Bees quickly recognize and long remember geometrical figures of the sort they encounter in nature, and at the flower they rely more on form and characteristic lines than on color shades (27). Frisch attributes the significance of color combinations in multicolored flowers to the role the patterns play in forming recognition features for bees.

Honey-bees, finally, can distinguish odors well and can remember them for days or even weeks (29). Bees conditioned to the fragrance of acacia visit this alone and are not in the least attracted by the odor of rose or lavender. Bees conditioned to the odor of orange-peel distinguish this from 43 other essential oils and confuse it only with bergamot and cedar oil. Foul smelling odors, however, evoked no response from the bees. Since bees can perceive colors at considerable distances but odors only when close at hand, Frisch has inferred that the role of fragrance is not to attract the pollinating bee, but rather to provide her with a most important means of discrimination once she has found the flower.

This hypothesis demands, of course, that the different kinds of flowers visited by bees should each possess a characteristic odor. Anton Kerner (33*a*, p. 203) has indeed pointed out that closely allied species of plants often have very different odors. He cited in this connection four species of *Daphne*, of which *D. alpina* is vanilla-scented, *D. striata* lilac-scented, *D. philippi* violet-scented, and *D. blagayana* clove-scented. A blind man could distinguish between certain species of *Orchis, Rosa, Syringa* and *Sambucus* by their odors, and in *Viola, Primula* and *Habenaria* one member of a pair of species is strong-scented, while the other has no scent.

The researches of Frisch place us in a position to understand certain older observations on bees. The experimental bouquets devised by the earlier workers (19, 22, 59) did not cause the bees to change from one kind of flower to another because they recognized differences in form. One example will suffice. Detto (22) accustomed some bees to heads of *Rudbeckia laciniata*. He then removed the disks of *Rudbeckia*, substituting disks of the (to his eyes) similar *Heliopsis laevis*. Notwithstanding the fact that bees were independently visiting *Heliopsis* at the time, the bees on *Rudbeckia* merely investigated the new disk florets but did not enter them.

Cases of bees going freely between different color forms of the same species are frequently recorded. Plateau (60) thus reported *Anthidium manicatum* and *Megachile ericetorum* to pass from pink to blue forms of *Salvia horminum* in his garden. Similarly *Bombus terrestris* crossed white and pink varieties of *Althaea rosea,* and blue and pink varieties of *Delphinium ajacis*. *Apis mellifica* crossed blue, violet, pink and white forms of *Centaurea cyanus*.

Darwin (21, ch. xi) reported similar instances: "Humble and hive-bees are good botanists, for they know that varieties may differ widely in the colour of their flowers and yet belong to the same species. I have repeatedly seen humble-bees flying straight from a plant of the ordinary red *Dictamnus fraxinella* to a white variety; from one to another very differently colored variety of *Delphinium consolida* and of *Primula veris;* from a dark purple to a bright yellow variety of *Viola tricolor;* and with two species of Papaver, from one variety to another which differed much in colour; but in this latter case some of the bees flew indifferently to either species, although passing by other genera, and *thus acted as if the two species were merely varieties*". (Italics of the reviewer).

When closely related subspecies or species of plants, differing by some well marked floral characters and not by mere color as in the foregoing examples, are grown together in a garden, the pollinating honey-bees remain in general on one kind of plant at a time and do not cross-pollinate them. This was the case in garden experiments with three subspecies of *Gilia capitata* (31). One of the subspecies, *Gilia capitata chamissonis*, possesses oval corolla lobes and creosote-scented nectar; the other two subspecies, *G. capitata capitata* and *G. capitata tomentosa,* have linear corolla lobes and sweet-scented nectar. The honey-bees in the garden, while freely circulating between the two linear-lobed subspecies, nevertheless demonstrated considerable constancy to either the oval-lobed or the linear-lobed group of gilias. Observations of the bees at the flowers showed that discrimination, involving positive rejection in the presence of the alien kind of flower, was being exercised, and suggested very strongly that the form of the corolla lobes and/or the odor of the nectar were in fact the determining factors.

When 145 progeny of the oval-lobed subspecies, *G. capitata chamissonis,* were grown the following year, it was found that these consisted of only 2.1% of hybrids with *G. capitata capitata* but 19.6% of hybrids with *G. capitata tomentosa.* In the absence of any significant difference in compatibility between the cross *G. capitata chamissonis* ♀ × *G. capitata tomentosa,* on the one hand, and *G. capitata chamissonis* ♀ × *G. capitata capitata,* on the other, it may be concluded that the difference between 19.6% and 2.1% contamination reveals a markedly lower degree of flower constancy between the former pair of subspecies as compared with the latter.

The significance of this relatively low degree of constancy between *G. capitata chamissonis* and *G. capitata tomentosa* may consist in the fact that *G. capitata tomentosa,* while possessing linear corolla lobes like *G. capitata capitata,* nevertheless approaches *G. capitata chamissonis* in size and other features of the corolla. The floral differences between *G. capitata chamissonis* and *G. capitata tomentosa* may lie at the threshhold of the powers of discrimination of the bees (see illustrations in 31).

When the completely intercompatible species, *Antirrhinum glutinosum* and *A. majus,* which differ in the size and markings of the corolla, were grown intermixed in a garden and open-pollinated by honey-bees, so little interspecific cross-pollination occurred that in the progeny of *A. glutinosum* only 2.9% contamination from *A. majus* was found (50). In the same progeny of *A. glutinosum,* however, 4.2% of the individuals showed contamination from a group of various hybrid derivatives of *A. glutinosum* × *A. majus,* differing from the species mainly in flower color and growing 400 feet away. These results point once more to the conclusion that perception of similarities in floral parts, much more than propinquity, forms the basis for the flower constancy of bees.

THE ORIGIN OF FLOWER CONSTANCY

Darwin (21) postulated that the instinct of flower constancy was established by natural selection, operating to preserve the bee of superior efficiency. A constant bee could work more quickly than an inconstant one, for, having learned how to insert her proboscis in one flower, her newly formed techniques would suffice for all other flowers of the same species. Plateau (61), disagreeing with this hypothesis, held that the advantage to the bee consisted not so much in saving of time as in saving of muscular fatigue during the transport of pollen loads. It does not appear that Plateau's suggestion is incompatible with that of Darwin. Dr. E. G. Linsley has pointed out in a communication to the author that natural selection will operate more directly on solitary bees than on such social bees as *Apis mellifica* where the pollen and nectar-collectors are non-reproductive.

Loew (42), viewing the problem in the larger perspective of flower-visiting insects as a whole, also held that flower constancy is adaptive. He distinguished, on the basis of correlated morphological and psychological characteristics, three stages of adaptation of insects to flower visits, as follows.

Allotropous insects are flower visitors which are only slightly adapted for pollination. They lack both structural adaptations and flower constancy. Their movements are irregular, and they feed on a variety of foods besides flowers. (COLEOPTERA: most flower-visiting beetles. DIPTERA: Muscidae, Empidae. HYMENOPTERA: many flower-visiting wasps, i.e., Vespidae, Ichneumonidae.)

Hemitropous insects are flower visitors which are partially adapted for pollination. They possess feebly developed structural adaptations and an intermediate degree of flower constancy. Their movements are on the whole regular and moderately skillful. (COLEOPTERA: a few beetles, i.e., *Nemognatha*. DIPTERA: Conopidae, Syrphidae, Bombyliidae. HYMENOPTERA: some wasps, i.e., Pompilidae, also some short-tongued bees, i.e., *Prosopis, Sphecodes*. LEPIDOPTERA: most flower-visiting butterflies and moths.)

Eutropous insects are flower visitors which are completely adapted for pollination. They possess highly developed structural modifications and habits of flower constancy, which enable them to efficiently rifle flowers and cross-pollinate them. (HYMENOPTERA: most flower-visiting bees. LEPIDOPTERA: Sphingidae.)

With respect to bees, Loew (41) proposed another classification which has had considerable theoretical value. Monotropic bees comprise those species of bees which visit the flowers of only a single species of plant (i.e., species of *Andrena*); oligotropic bees are species that visit a few allied species of plants; and polytropic bees go indifferently to very diverse flowers. Since many bees visit only one species of flower for pollen but many other kinds of flowers for nectar, Robertson (64) introduced the parallel terminology (monolectic, oligolectic and polylectic) to refer specifically to the behavior of the pollen-collecting females. These distinctions apply at the level of species of bee and are related only indirectly to the flower constancy of individual bees. *Apis mellifica* is a polytropic bee, for example, in that the species as a whole may be found working on unrelated species of plants: yet individual workers, as we have seen, do not often stray from a single species of plant until they have exhausted the supplies of nectar or pollen on it.

The long polemic between Robertson and Lovell on the origin of oligotropism in bees therefore concerns us only indirectly in this review. Robertson (62) believed that the oligotropic habit originated as a result of competition between different species of bees, with natural selection intervening in favor of those species which are the least competitive, viz., facultatively oligotropic, in their food habits. "My view is that the bee fauna is all that the flora will support, that there is constant competition between the bees [read "species"], and that natural selection favors those which are the most diversified, i.e., the least competitive in their food habits". Lovell (45) disagreed with Robertson's premise that competition exists between different species of flower-feeding bees. He held that plants produce nectar far in excess of the bees' requirements. Without attempting to pass final judgment on the correctness of Lovell's assertion that competition for nectar does not exist in nature, it must be admitted that he at least did not establish the absence of competition for pollen.

It seems probable on *a priori* grounds that a necessary precondition for the origin of flower constancy in bees must have been the rise of angiosperms with concealed nectar and pollen. In a world of polypetalous flowers with numerous stamens and freely accessible nectar or pollen it is difficult to imagine the existence of any selective advantage for the confinement of visits to one particular kind of flower, and the inhabitants of these generalized flowers in the modern flora do not possess instincts of constancy (31, 42). The discussion of the origin of flower constancy can not be separated, therefore, from a discussion of the mutual evolution of angiosperms and bees.

FLOWER CONSTANCY AND THE EVOLUTION OF ANGIOSPERMS

Most studies on flower constancy have of necessity been carried out with the honey-bee, an Indomalasian species which has attained world-wide distribution only within historical times, or else with native oligotropic bees on introduced plants. The parallel data for bees in the wild, where known, however, indicate that they too exhibit a high degree of flower constancy. It is legitimate, therefore, to consider some of the possible consequences of the instinct of flower constancy of wild bees in the evolution of the angiosperms.

We may consider our subject, for convenience, in relation to two fundamental patterns of evolution, namely progressive evolution, or the sustained changes in the characteristics of populations, and species formation, or the segregation of variations into distinct populations (see 66).

PROGRESSIVE EVOLUTION. The primitive *Magnolia* type of flower possesses numerous separate petals with little or no color and numerous separate stamens, and usually lacks well developed nectaries (12). Mesozoic prototypes of these flowers may very well have been pollinated, as many of their modern representatives are, by beetles of little skill and no flower constancy (23, 32*a*). The insect-pollinated angiosperms of Mesozoic times must in any case have been inefficiently pollinated in the absence of any of the more specialized groups of flower-visiting insects during that era.

The consequent failure of seed set would have placed a selective value on any changes in the flowers tending to confine the insect visitors to one kind of flower at a time, for less pollen would then have been wasted on random interspecific visits and the reproductive potential of the plants would have been increased. Mutations causing the petals to grow up as a fused corolla tube around the stamens might have fulfilled this condition. Fewer types of insects would find entrance to a tubular corolla than to an open flower. Those insects which could feed on tubular flowers, furthermore, would encounter less competition for the available supply of nourishment, and so would be favored by the development of habits enabling them to remain constant to such types of flowers. The instinct of flower constancy may thus have been preserved by natural selection, both for its advantage to the plant, which became better pollinated, and for its advantage to the insect, which became better fed.

The results of the screening process exercised by the tubular structure of the corolla are apparent today in the different types of insects which inhabit sympetalous and polypetalous flowers. Müller has estimated that in the Alpine flora of Europe, beetles, wasps, flies and other unspecialized and inconstant insects, which constitute 86% of the insect species on flowers with open nectar, form but 37% of the visitors to flowers with concealed nectar (55, p. 490).

The fusion of the petals into a tubular corolla, which made it possible for the plant to leave off the production of numerous stamens and to store and conceal nectar at the base of the corolla tube, must have tended to restrict the pollinating visitors to the ancestors of the bees, long-tongued flies, moths and butterflies (8). These groups of insects, which feed on tubular flowers and possess long tongues and habits of flower constancy, appeared in geological history at the beginning of the Tertiary period (see 32*b*). Most, viz. 86%, of the sympetalous families of angiosperms that are known in the fossil record at all likewise made their first appearance in the early part of the Tertiary (see 61*a*). By early Tertiary time, therefore, a course of mutual evolution involving sympetalous flowers and long-tongued and flower-constant insects, among them bees, was well established.

A still more specialized method of cross pollination of plants, was attained with the development of special floral mechanisms accessible only to certain kinds of bees and designed so that the stamens and stigma of the flower would touch the bee in some one spot (8). The success of such bee flowers depended upon a combination of factors, among them the coloration of the flower, the presence of a landing platform for the bees, and the efficient operation of the floral mechanism. Those groups of angiosperms which had entered upon a course of floral evolution in relation to pollination by bees would accordingly have evolved a brightly colored corolla tube with an extended lower lip and a reduced number of specialized stamens.

The development of these floral characters, which are the elements of a successful bee flower, will have occurred repeatedly in different phyletic lines of plants, just as the transition to flower constancy, long tongues and body hairs has occurred repeatedly in different orders of insects. The same combination of floral characters—two-lipped tubular corollas colored blue or yellow and containing sweet-scented nectar, a reduced number of stamens, and a superior ovary composed of several fused carpels—has arisen independently in several different phyletic lines. This combination of characters is present in the more or less distantly related families Labiatae, Verbenaceae, Papilionaceae, Polygalaceae, Scrophulariaceae, Violaceae, Fumariaceae inter alia.

SPECIES FORMATION. The flower constancy of bees, based on the perception by the bees of likenesses and differences in the form, color and odor of flowers, constitutes a mechanism for the isolation of populations of bee plants which differ in floral characters (31). The operation of this instinct of flower constancy has two important consequences in the process of species formation in bee plants.

The principal evolutionary role of the isolating mechanism based on the pollinating behavior of bees is to augment the efficiency of geographical speciation in bee plants (31). It follows that angiosperms which are pollinated by bees will possess a more effective means of evolutionary divergence than angiosperms which are pollinated by inconstant insects (promiscuous plants). More rapid species formation should thus occur in bee plants than in promiscuous plants. A survey of the flora of Southern California, which is an area very rich in endemic bees, did in fact reveal a significantly higher number of species per genus in bee plants than in promiscuous entomophilous angiosperms—5.9 and 3.4, respectively (31).

It also follows from the nature of the isolating mechanism imposed by the flower constancy of bees that bee plants will in general be more differentiated into species on floral characters than promiscuous angiosperms. A survey of the taxonomic characters which have been used in a wide variety of floras and monographs to separate pairs of species in the ultimate divisions of diagnostic keys for several hundred genera, showed that floral characters do indeed predominate in bee plants. Forty per cent of the taxonomic characters between closely related species of bee plants pertain to the petals and other inner floral parts; the corresponding figure in promiscuous entomophilous angiosperms is but 15% (31).

Bee pollination, therefore, has brought about both a convergence in the structural characters of flowers related to the floral mechanism, and a diversification in the form, color and odor of flowers. The latter category of floral characters, upon which the taxonomy of bee plants is to a large extent based, has arisen as the recognition features of bees. Those groups of angiosperms which have imposed flower constancy on bees and other insects by such developments as the tubular corolla have undergone a diversification of flower forms, colors and odors as a necessary concomitant to the practise of flower-constant pollination (27, 31).

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LEAF XEROMORPHY AS RELATED TO PHYSIOLOGICAL AND STRUCTURAL INFLUENCES

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INTRODUCTION

The greater part of Maximov's masterpiece, The Plant in Relation to Water, based on original research and on more than 450 references, was ready for the press in 1925. While few investigators have recently considered the specific problems of drought resistance and xeromorphy, several significant contributions clarify earlier observations which were not understood 25 years ago. White's work on root pressure (1938) revealed why water moves in one direction through plant cells. Turrell's measurements of internally exposed leaf surface in xerophytes (1936) explained Maximov's observation that in the presence of adequate moisture transpiration is higher in xeromorphic than in mesomorphic leaves. Boon-Long (1941) interpreted the effect of high osmotic pressure in retarding water loss through decreasing cell permeability. Popp (1926), Shirley (1929) and Turrell (1940*a*, *b*) evaluated critically the influence of light intensity on leaf morphology. Wylie's studies of leaf structure in mesophytic and tropical dicotyledons (1939, 1943, 1946) suggest similar problems of transfer and contrasting surface-volume relationships in xeromorphic leaves. Mothes (1931, 1932) detected the effect of a nitrogen deficiency in contributing to xeromorphy. Other investigations of mineral nutrition have shown the effects of a deficiency or an excess of an element on water relations and structural modifications (Prianischnikov, 1928, 1935; Lundegardh, 1932; Hoagland and Broyer, 1943, *et al.*). Hoagland and Broyer (1936) determined the mechanism of absorption of these elements which so significantly affect the structure of many plant parts.

While no better explanation of survival in xerophytes is known than the one Maximov formulated, knowledge relating directly or indirectly to xeromorphy has been extended since his work.

STRUCTURAL MODIFICATIONS OF XEROMORPHIC LEAVES

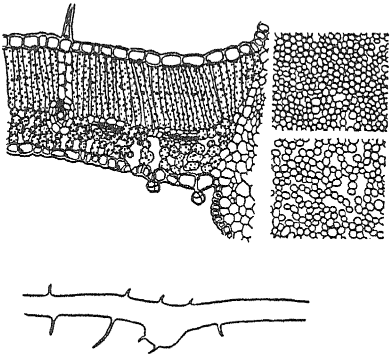
Xerophytes are dry habitat plants with transpiration decreased to a minimum under conditions of water deficiency (Maximov, 1931). Certain tissues of xerophytes, particularly those of the leaf, become altered structurally in relation to environment. The most obvious characteristic of xeromorphic leaves is the lower ratio of surface to volume (MacDougall and Penfound, 1928; Weaver and Clements, 1929) (Fig. 1). Reduced external area is accompanied by certain modifications in internal leaf structure: decreased cell size, thicker cell walls, a more compact network of veins, a higher stomatal frequency, and palisade strongly developed at the expense of spongy mesophyll (Schimper, 1903; Maximov, 1929; Weaver and Clements, 1929). Trichomes are also characteristic of many xeromorphic leaves, and water storage tissue forms in the xeromorphic succulent leaf type.

Certain of these modifications establish conditions favorable to increased photosynthesis and in many instances to increased transpiration as well (Maximov,. 1931). Xerophytes, however, have in common an internal resistance which restricts water loss in time of drought and an ability to endure desiccation (Maximov, 1931).

The response of leaf morphology to external environment is recognized in the assumption that climatic agencies in areas having dissimilar vegetation can be compared through leaf-size groups constituting the flora (Raunkiaer, 1887).

ENVIRONMENTAL FACTORS WHICH CONTRIBUTE TO GENERAL LEAF XEROMORPHY

WATER DEFICIT. Leaf xeromorphy may result from excess transpiration or low absorption and the consequent water shortage which limits stretching growth (Schimper, 1890; Transeau, 1904). Cell walls thicken from accelerated conversion of polysaccharides into anhydrides or wall materials (MacDougal and Spoehr, 1918) deposited over the limited cell surface. Veins form a denser network, not being widely separated by laterally expanding epidermis



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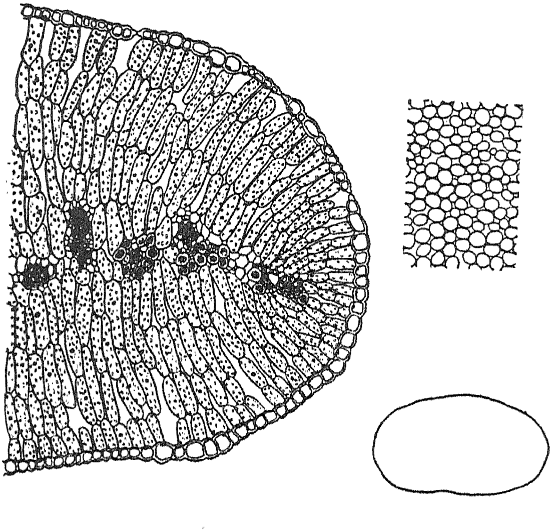


GREGGIA CAMPORUM LINEARIFOLIA

Fig. 1. Transverse and paradermal sections and trans-sectional diagrammatic outline of mesomorphic (above) and xeromorphic (below) leaf types. Note that while within a species cell size may be smaller in xeromorphic than in mesomorphic forms, leaf cells of xerophytic species are unusually large (Shields, 1951).

and spongy mesophyll. The increased number of stomata per unit area results from limited enlargement of epidermal cells (Weaver and Clements, 1929). The plastic *Rumex acetosella* has one cell layer of palisade when grown under moist conditions, but when grown on dry sand two or three palisade layers form, and the leaves become thicker, smaller in size and revolute margined (Transeau, 1904). The amount of transpiration relative to water supply rather than absolute water loss leads to these structural modifications (Schimper, 1903), high evaporation influencing tissue proportions only when an internal water deficit exists (Cain, 1933).

HIGH LIGHT INTENSITY. Since the optimum light intensity for growth in leaf thickness is considerably higher than for increased surface, strongly illuminated leaves are small and thick (Schimper, 1903). Shade leaves of *Rhus toxicodendron* growing in moist dunes of a maple forest have five times the area of those from a sandy level with few trees (Turner, H., 1923), and compactness in spongy tissue varies directly with xerophily of habitat. *Vinca rosea* and *Nerium oleander* leaves are more xeromorphic when grown under 300-watt Mazda lamps than under similar 200-watt lamps (Turrell, 1940*a*). Twenty percent of summer sunlight favors maximum leaf expansion (Shirley, 1929), and above 50 percent ordinarily limits enlargement (Weaver and Clements, 1929). The optimum light intensity for the production of dry weight, however, increases with plant age (Combes, 1910). The percentage of dry matter in tops, the ratio of dry roots to dry shoots, density of growth and stem strength, as well as leaf thickness, increase with light intensity (Shirley, 1929). Plants grown in sunlight develop in the leaf mesophyll 77 percent more cell substance than do leaves of shade plants of the same species (Hormann, 1927). Large amounts of the blue end of the spectrum also induce xeromorphy, while removal of the blue portion, necessary for normal vigorous growth, stimulates mesomorphic development (Popp, 1926), but the decreased light intensity from removal of the blue fraction may contribute to this mesomorphy. Microphyllous plants as a rule are light demanding and succumb in competition with forest types if moisture conditions are sufficiently favorable (Thoday, 1931).

Light indirectly affects both transpiration and carbohydrate synthesis. Depending upon the evaporating power of the air, the fraction of transpiration resulting from the direct effect of radiation varies from 38 to 81 percent (Martin, 1935). Since light intensity rises in advance of transpiration and of all other environmental factors and falls before transpiration or with it, radiation may be the primary causative factor in cyclic changes in water loss during the day (Briggs, 1916). The daily curves of cuticular transpiration in xerophytic *Rhododendron* and *Hedera* spp. follow the curve of light intensity more closely than the curve of water evaporation from an atmometer bulb (Mittmeyer, 1931). This is also true of total transpiration in sun leaves, possibly because the plant is more sensitive to light and an atmometer to wind (Maximov, 1929). Solar radiation is responsible for the great difference between day and night transpiration (Maximov, 1929). Even a 6.5-candlepower light accelerates water loss (Wiesner, 1877). Transpiration in *Hedera helix* and *Prunus laurocerasus* transferred from dark to light increases 30 percent (Darwin, 1914). Yet in 25 of 60 European plant species examined the stomata remained open at night (Stahl, 1919).

Transpiration is chiefly influenced by stomatal movement and soil water content (Maximov, 1929). Stomata of barley, wheat, oats and rye open in the light and close in the dark (Gray and Peirce, 1919). Stomata in species of *Poa, Festuca* and *Agrostis* open to a maximum from 9 to 10 A.M. and completely close at night (Carroll and Welton, 1937). Those of *Coffea arabica* remain open in dull weather or in shade, but in direct sunlight close between 10 A. M. and 4 P. M. from the action of light on the stomata and not from water relations of the leaves (Nutman, 1937). Stomatal behavior is such in eight trees and shrubs examined that the maximum standard rate of water loss occurs early in the day, usually between the hours of 8 and 10 A. M (Meyer, 1927).

Water availability influences the effect of light on transpiration. With adequate soil moisture transpiration in oaks is continuous during the day and ceases at night, but when soil moisture is near the wilting coefficient stomata may remain closed all day (Yocum, 1935). In castor bean soil moisture rather than light intensity conditions the ratio of average transpiration to xylem area (Penfound, 1932). The time of maximum opening of stomata on citrus leaves is four hours after sunrise under moist conditions, but becomes progressively earlier as drought increases (Oppenheimer and Elze, 1941; Mendel, 1945). Stomata of xerophytes, however, close only in very intense light (Mittmeyer, 1931).

The role of light in regulating stomatal apertures may be controlled by variation in the hydrogen ion concentration of the guard cells which in turn governs turgor (Scarth, 1932). Light decreases acidity in the green guard cells, turgor is increased, and the stomates open (Scarth, 1932). Light may also affect permeability of the protoplasm in guard cells. Guard cells bounding closed stomata are filled with starch which changes to sugar as the stomate opens (Lloyd, 1908), the osmotic pressure rising simultaneously from 10 or 20 to 90 or 100 atmospheres (Iljin, 1914). Guard cells plasmolyze when a leaf is placed in salt solution, but regain turgidity as the salt penetrates, apparently inducing starch hydrolysis (Iljin, 1922). Transpiration in green areas of *Hedera* and *Pelargonium* leaves transferred from dark to light is double that in white leaf parts on the same plants (Leclerc du Sablon, 1913). In variegated leaves normally functional stomata may be entirely devoid of green plastids (Kummler, 1922).

There is also a non-stomatal control of transpiration from retardation of absorption, retreating of water menisci within the pores and diminishing pressure of water vapor at the cell surfaces (Maximov, 1929). Water, as well as dry matter, residual ash and potassium, in three to seven weeks old maize leaves increases in the early morning, falls to a minimum around 11 A. M., and rises again in the afternoon (Penston, 1938).

Increased light, decreased water or a combination of the two result in reduced leaf surface and greater leaf thickness (Clements, E., 1905). Upper leaves, farther removed from the water supply and exposed to higher light intensity, are more xeromorphic than lower leaves of the same plant (Zalenski, 1904; MacDougall and Penfound, 1928). Bright light and wind, however, are of secondary importance compared to deflection of the water current by transpiration of lower leaves (Maximov, 1929), limiting the water supply while upper leaves are still in the bud (Zalenski, 1904). The water content of successive leaves diminishes with increasing height to near the apical region, maximal water percentage being in the lowest leaves not past the age of functional activity (Yapp and Mason, 1932). Increasing severity of conditions encountered in upward growth accelerates transpiration to the amount of 6.06 cc./m./day, to four meters in pine stands and 3.55 cc./m./day, to 6.1 meters, in aspen groves (Gates, 1926). While there is no constant relationship between cell length and stem length, in both tomatoes and zinnias the first ten cells behind the promeristem are shorter at successive stages in the plant's ontogeny (Bindloss, 1942), apparently from a diminishing water supply. As the position of the apple leaf approaches the shoot apex palisade cells become more elongate and compact and make up a greater percentage of the mesophyll (Cowart, 1936). In alfalfa the areas and internal-external surface ratios of primary, late primary, secondary, tertiary and quaternary leaves are, on the average, successively smaller (Turrell, 1942). Differences in thickness of leaves from the south periphery and center leaves on the same tree are usually greater than corresponding variations in leaves of mesophytic anad xerophytic forms of a species (Gates, 1917; Hanson, 1917). The pattern of foliar development in aster may be related to the concentration and distribution of auxin (Delisle, 1938), but these, in turn are influenced by water and light.

The minimal water content occurs in the sunflower leaf when one-third to two-thirds normal size (Yapp and Mason, 1932), water shortage at this critical stage apparently contributing to the changed anatomical elements in the ascending leaf series. In trees and shrubs, however, conditions of illumination and transpiration under which buds were formed the previous year appear to predetermine stomatal size and number, extent of conducting tissue and thickness of epidermal walls (Nordhausen, 1903). The number of mesophyll layers of a shade leaf probably is determined in the bud (Smith, 1934). Marginal dentation of *Riparia glabra* and *R. du lot* occurs before budding begins (Rodrigues, 1941). Yet, removal of lower buds of *Tilia europaea* causes the remaining one to develop abnormally large leaves, suggesting that the total cell number is not established at an early date (Ewart, 1906). The larger leaf, however, might result from increase in cell size rather than in cell number. In *Coldenia hispidissima,* cell number and arrangement are much the same in young and old leaves (Fig. 2), normal leaf enlargement consisting of increase in size of existing cells (Shields, 1951). Removal of the inflorescence and axillary buds of tobacco causes an appreciable increase in leaf size on the upper one-third of the stalk with a proportional increase in size of epidermal aand palisade cells (Avery, 1934).

Modifications in sun leaves are attributed to a water deficit in tissues at some time during formation (Soding, 1934). When *Manihot utilissima* is transplanted from a lighted place to shade narrow lobed leaves become broad lobed (Graner, 1942). Altering the availability of water by adding NaCl to the soil induces characteristics of sun leaves in all foliage of *Tilia cordata, Fraxinus excelsior* and *Acer platanoides* (Soding, 1934). Given an ample water supply, however, sun plants of *Helianthus* have larger leaves, larger epidermal cells, thicker mesophyll and more numer-

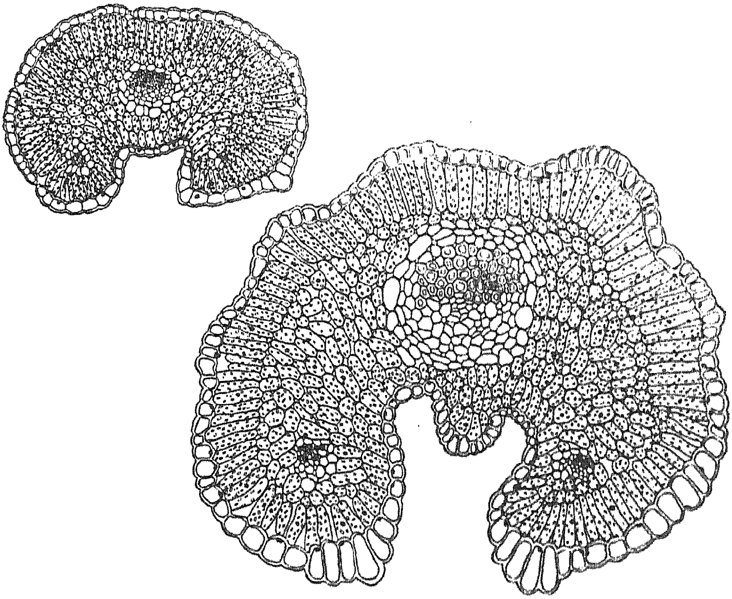


FIG. 2. Cell arrangement and cell number are much the same in young (left) and old leaves (right), enlargement consisting of increase in cell size rather than cell number (Shields, 1951).

ous stomata than shade plants (Penfound, 1931). Mature shade leaves of *Cornus florida rubra* and *Acer platanoides* are smaller, as well as thinner, than comparable leaves from opposite unshaded shoots (Isanogle, 1944). Cell layers are the same in leaves having ten percent up to full intensity of sunlight, anatomical differences being the result of cell elongation (Isanogle, 1944).

The 15 taxonomic varieties of *Ranunculus scleratus* are believed to represent responses to different amounts of water (Folsom, 1918). It has been demonstrated in 200 genetically distinct groups that the same individual plant produces a more xeromorphic leaf type in alpine regions than in lowlands (Coulter, Barnes and Cowles, 1931). Xeromorphy in *Senecio* spp. in mountain ranges of equatorial Africa is correlated with altitudinal distribution of the individual species (Hare, 1941). No difference was observed, however, in size or anatomy of leaves and bud scales of *Populus tremuloides* collected at 5800′ and 9000′ in Colorado (Shope, 1927), probably because in this case moisture and temperature conditions were not more unfavorable at the higher altitude.

Ordinarily leaves of woody sprouts or saplings are larger than those of older trees, but certain young apricot trees were found to bear smaller leaves (Passecker, 1940). The average area of one blade surface for ten mature leaves from a cottonwood sapling five feet high growing in a calcium sulfate deposit was 18 square centimeters compared to a mean of 110 square centimeters in leaves of a nearby old cottonwood under equivalent conditions (Shields, 1951).

Increase in leaf blade area may accompany decreased surface tension of the leaf sap when energy gained as surface tension is reduced, accelerating chemical reactions on cell interfaces and resulting in increased area (Hercik, 1927). Possibly plant parts follow the compound interest law, the amount of material added in growth depending upon the amount already present (Blackman, 1919).

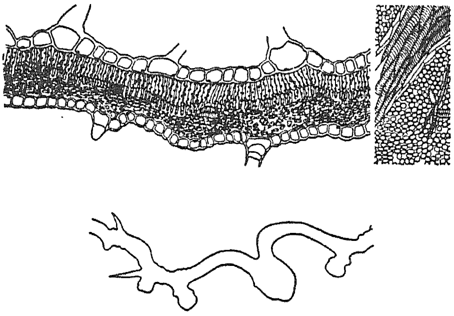
NITROGEN DEFICIENCY. In water cultures of *Nicotiana rustica, Zea mays, Coleus* and *Tradescantia albiflora* low nitrogen, as NH_4NO_3, induces xeromorphy, greater osmotic value and higher ash content percent of fresh weight, but less percent dry weight (Mothes, 1932). Low nitrate produces a relatively xeromorphic plant with thick cell walls, more mechanical and sclerenchymatous tissue, heavy cuticle and thick leaf epidermis (Kraus and Kraybill, 1918; Welton, 1928; Schneider, 1936). A high salt content is correlated with succulence, and high salt with low nitrogen produces a xeromorphic succulent leaf type (Mothes, 1932). Differences in the nitrogen supply and salt content may figure in the development of sun and shade leaves (Mothes, 1932). In wilted plants water and soluble nitrogen compounds are removed from older, lower leaves and transported into younger leaves at higher insertions (Mothes, 1931). Work with eight typical moor plants, however, indicates that xeromorphy results from a number of factors and not specifically from nitrogen starvation (Marthaler, 1939).

INFLUENCES RESPONSIBLE FOR THE GREATER DEVELOPMENT OF PALISADE

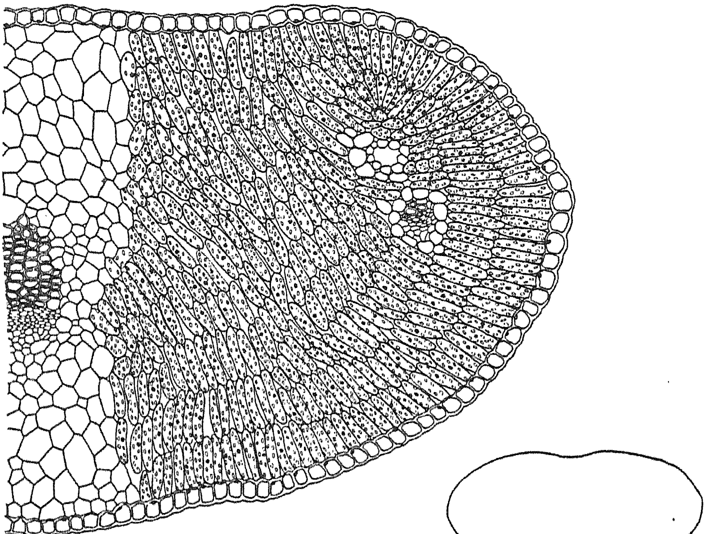
The increased thickness and smaller size of xeromorphic leaves are associated with the greater development of palisade, elongating vertically at the expense of laterally expanding spongy mesophyll (Fig. 3). High light intensity and an internal resistance to flow, resulting from a water deficit, apparently contribute to the formation of palisade tissue.

HIGH LIGHT INTENSITY. Intense illumination causes chloroplasts to aggregate into a compact clump, the majority in shaded positions, leaving the cell walls altogether (Frank, 1872) through passive transportation by the cytoplasm (Haberlandt, 1928). In weak or partially intercepted sunlight chloroplasts shift to the radial walls (Frank, 1872). The tension caused by laterally arranged chloroplasts may increase the horizontal cell axis at the expense of the vertical axis (Clements, E., 1905; Jost, 1907). This action of light on chloroplast arrangement and the consequent change in shape of cells containing them may influence leaf form, direct radiation causing chlorenchyma to elongate perpedicular to the surface (Weaver and Clements, 1929). Mechanical shock or injury, a water deficit, fluctuations in temperature or chemical stimulation, however, may also cause chloroplasts to shift from paradermal to radial walls and even to the center of the cell (Haberlandt, 1928).

Deeper palisade probably effects improved illumination of a



CUCURBITA FOETIDISSIMA



SENECIO SPARTIOIDES

FIG. 3. Transverse sections illustrating the contrasting combinations of volume and external surface in mesomorphic (above) and xeromorphic (below) leaves. In this *Cucurbita foetidissima* leaf, which exposed 282 sq. cm. of upper leaf surface and measured 150 μ from the upper through the lower epidermis, the surface-volume ratio was 133.2. In the *Senecio spartioides* leaf, exposing 4.65 sq. cm. total external surface and measuring approximately 1602 μ in thickness, the surface-volume ratio was 21.2 (Shields, 1951).

larger volume of cells (Thoday, 1931), but palisade is least developed in shade leaves most in need of light. Chloroplasts in palisade tissue behind a highly light diffusing epidermis are subjected to a light intensity which is directly proportional to cell diameter, narrow palisade protecting against intense illumination (Nielsen, 1940). Xeromorphic leaf structure in bog, heath and dune plants deficient in nitrogen may be an adaptation against too intense illumination of chloroplasts (Nielsen, 1940). In 19 of 37 plants with typical hereditary white mottling of the foliage, however, the classical palisade structure is present in the absence of chloroplasts (Conti, 1934). Palisade inception occurs independently of illumination in the darkness of the leaf bud, and most leaves, even in deepest shade, form some palisade (Haberlandt, 1928). Light intensity, however, apparently influences the location of this tissue most active in photosynthesis. In many leaves illuminated from both sides the entire mesophyll is composed of palisade, sometimes with a middle layer of intermediate cells. Palisade occasionally develops, however, immediately within the lower epidermis in shade leaves (Haberlandt, 1928). In *Iris japonica* leaves which normally have a palisade layer above and a spongy parenchyma below with stomata on the lower surface only, reversing the leaf position during development causes a reversal of tissues, but the effect of light on the dorsiventral condition is slight (Imamura, 1931).

Palisade is rare in stems which bear typical leaves. In certain species of *Polygala*, however, peripheral strata of stem cortex consists of palisade cells (Holm, 1929). In essentially leafless stems, as *Ephedra torreyana* (Fig. 4) and *Allenrolfea occidentalis*, deeper layers of stem parenchyma regularly elongate at right angles to the main axis, forming cortical palisade (Shields, 1951).

In English ivy starch accumulation under increased light conditions, and the subsequent conversion of starch to sugar, results in higher osmotic pressure and consequent cell elongation, forming palisade (Watson, 1942). In citrus leaves the ratio of depth of palisade tissue to leaf thickness is nearly constant for each of several species examined (Halma, 1929).

INTERNAL RESISTANCE TO FLOW. As water supply is depleted there develops a resistance to flow, accompanying a high osmotic pressure, which is the result of purely physical conditions in the

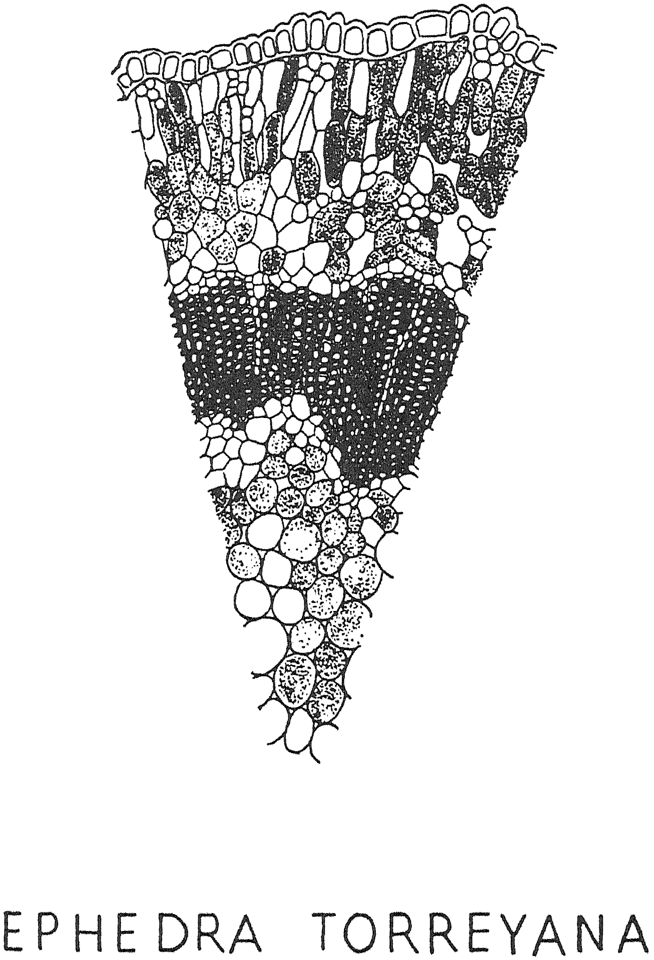


FIG. 4. Showing cortical palisade which often appears in essentially leafless stems.

plant and soil (Shreve, 1923; Emerson, 1947). Resistance to flow in the leaf is apparently greatest in passage through protoplasts of the mesophyll (Mer, 1940), and particularly from one cell to another (Maximov, 1929) rather than in minor veins. Yet when veins of *Ginkgo biloba* leaves are cut, water travels laterally through mesophyll cells with sufficient speed to maintain life and rapidly enough to supply cells in certain cases to a distance of 2.6 mm. (Shull, 1934). In ivy leaves resistance to lateral flow is greatest in the spongy mesophyll (Ursprung, 1935). Intercellular spaces handicap diffusion through the mesophyll, and particularly the lateral discontinuity of palisade in mesomorphic leaves limits transfer through this zone in the plane of the blade (Wylie, 1943). The centric type of xeromorphic leaf, however, in which palisade cells radiate around central vascular bundles, reduces resistance in the mesophyll to a minimum (Thoday, 1931). Decreased internal resistance in xeromorphic leaves is more significant than reduction of external surface, which is often illusory (Thoday, 1931). Elongate palisade cells may facilitate transfer of food materials (Transeau, 1904), also, under the stress of a reduced water supply in xeromorphic leaves.

The relative volumes of sponge to palisade in groups of tropical and subtropical leaves yields a ratio usually related to mean vein spacing in the blade (Wylie, 1946). In mesophytic leaves increased amounts of palisade force veins closer together, while a larger proportion of spongy mesophyll favors their wider separation (Wylie, 1939). The average area of vein islets is smaller in leaves from older trees than in leaves from young trees (Tellefsen, 1922), which are ordinarily more mesomorphic in other respects as well. In xerophytes none of the veins are widely separated laterally, presumably because of limited expansion of sponge and epidermis in this plane (Weaver and Clements, 1929). A shift in the ratio of palisade to spongy mesophyll is automatically accompanied by an adjustment between vein frequency and size of intervascular areas. Since xeromorphic leaves are well vascularized, resistance to flow must originate outside the conducting system.

In *Acer pseudoplatanus* cambium in the leaf blade produces xylem only while the leaf is extending, no new veinlets developing after expansion ceases (Eliot, 1933), irrespective of changes in the environment which tend to deplete the water supply. In *Antirrhinum,* in contrast to the situation in *Crataegus* and *Solanum,* the genetic constitution of the epidermis apparently determines leaf form (Schiemann, 1940). The leaf epidermis of 46 mesomorphic species contains over 20 percent of the blade tissue and seems to function in water conduction in areas between veins when closely connected with smaller veins by vein extensions which, for ten species, appeared along 58 percent of the total vein length (Wylie, 1943). Possibly epidermis influences leaf form, not only by lateral expansion, but also by supplying a significant fraction of total water. One stage in the growth of plum leaf buds is marked by stretching of the upper epidermal cells when the leaf flattens out, accompanied by continued division in the mesophyll. The frequency of cell division among palisade cells then becomes greater than in the spongy mesophyll, and the intercellular space system develops in this tissue as a result (Tetley, 1932).

Under conditions of water shortage *Rumex acetosella* forms drops of resin or oil in the epidermis and cells adjacent to bundles (Transeau, 1904), possibly inhibiting transfer. Xeromorphy in evergreens is correlated with resistance to flow by the wood, and plants in which conductivity is least impaired are least xeromorphic (Thoday, 1931). A water deficit stimulates production of not only palisade but also other elongate cells, including trichomes and root hairs (Yapp, 1912).

LEAF XEROMORPHY AS RELATED TO

WATER LOSS. Decreased leaf size, an almost universal modification in xerophytes, is commonly regarded as an adaptation which reduces transpiration. There is enough correlation between small-leaved plants and dry situations to lend color to this view, but shoots with pinoid, ericoid or cupressoid leaves are usually abundantly supplied with leaves, large numbers compensating for small size (Warming, 1925; Thoday, 1931). The total externally exposed leaf surface of certain conifers is often much greater than in dicotyledons, and north temperate Coniferae need and expend as much water as dicotylous trees (Groom, 1910).

The ratio of internal to external surface is low for shade leaves, equaling 6.8 to 9.9, and high for xeromorphic sun leaves, equaling 17.2 to 31.3 (Turrell, 1936). The greater internally exposed surface results from a more extensive development of palisade and is probably responsible, in part, for the transpiration rate in xerophytes (Turrell, 1936), which is high under normal conditions (Schratz, 1931). The internal-external surface ratio is moderately but positively correlated with volume of the intercellular space (Turrell, 1940*b*).

Sun leaves of a variety of plants transpire more than shade leaves (Mittmeyer, 1931), often two to three times as much (Geneau de Lamarliere, 1892; Hasselbring, 1914). Shade plants of *Helianthus* and *Impatiens* transpire 50 percent less than sun plants (Meyer, W., 1934). In *Tilia, Acer* and *Ampelopsis* transpiration of sun leaves is greater than in shade leaves, both with and without water, and the degree of wilting of sun and shade leaves does not differ (Kisselew, 1927). In *Syringa* and *Caragana,* in which shade leaves wilt noticeably faster than sun leaves, transpiration of sun leaves is greater with a water supply and less without it (Kisselew, 1927). Sun leaves of the broad leaved evergreen *Olea europaea* transpire from three to ten times as much as shade leaves of the same species under normal conditions and two and one-half times as much as shade leaves under abnormal conditions (Bergen, 1904). Transpiration is neither negatively nor positively correlated with growth (Shantz, 1927).

In *Ipomea* leaves remaining after, or developing subsequent to, removal of leaves below them, stomatal equipment is significantly increased (Alexandrov and Alexandrova, 1927). There is no relation between the amount of transpiration and the number of stomata or length of the stomatal pore (Muenscher, 1915), but more numerous and smaller stomata tend to increase transpirational capacity (Yapp, 1912).

The absolute rate of transpiration at 6000′ does not differ from that at lower altitudes (Kochanovsky, 1926). Alpine plants appear to transpire on an average about as much water as those of the plains (Szmykiewicz, 1927), but data on transpiration at high altitudes are contradictory (Maximov, 1929).

TRANSLOCATION. Small leaves may offer better water economy in that all parts of the mesophyll lie within a short distance of veins (Thoday, 1931). In *Spiraea* the parts of leaf farthest from main channels of supply are the first to shrivel in drought (Yapp, 1912). Toward the tip a sunflower leaf withers more and sooner and recovers most tardily (Thoday, 1931). In lobed leaves the most vulnerable parts appear to be omitted (Thoday, 1931). The primitive angiosperm leaf was presumably lobed (Sinnott and

Bailey, 1915). Dicot leaves and leaflets with entire margins are predominantly in lowland-tropical regions; those with non-entire margins predominate in mesic cold-temperate areas (Bailey and Sinnott, 1916) where water is less readily available. Peripheral leaves of deciduous trees, as maple and elm, are more deeply lobed or more prominently toothed, as the case may be, and are smaller than leaves from the center of the same tree (Hanson, 1917). *Larrea tridentata,* the most successful and conspicuous xerophyte in North American deserts, produces prodigious numbers of bifoliate leaves believed to have been derived from a simple mesic ancestral leaf type (Runyon, 1934). Possibly in this case also the most vulnerable parts were omitted in adapting to xeric habitats. Given plenty of water, creosote bush behaves like an ordinary mesophyte (Spalding, 1904), but the leaf size and shape, not being plastic, do not change.

Anatomical differences in certain desert plants cannot account for differences in behavior (Shreve, 1923). Drought-resistant and partially expanded *Larrea tridentata* leaves which withstand a water content to dry weight ratio as low as 0.5 are anatomically similar to mature leaves on the same branch which cannot endure a moisture content much below a level characteristic of leaves of mesophytic woody plants (Runyon, 1936). This physiological difference is evidently related to conditions obtaining during the time the leaves are emerging from the bud (Runyon, 1936). A limited water supply increases drought resistance, evidenced by wilted buckwheat and sunflower plants contrasted with controls (Tumanov, 1927).

SHOOT-ROOT RATIO. In addition to transpiring surface, an estimate of total structural xerophytism must consider absorbing surface and resistance to flow from the root to the leaf (Thoday, 1931). Root systems may increase the water supply by more vigorous development or by deeper penetration of the soil (Iljin, 1916). Nineteen species under semi-arid conditions, however, developed fewer laterals than aquatic plants (Dittmer, 1948). High light intensity, which in most species causes diminution of stem and leaf size, accelerates root growth (Lubimenko, 1908). In sweet clover either cool temperature or a low moisture supply increases the proportion of total plant weight in the roots (Smith, T. J., 1942). The absorbing surface of corn, exclusive of root hairs, amounts to 1.2 times the surface of above-ground parts in moist soil, and is 2.3 times as great in dry soil (Weaver, 1925), root area increasing as stem and leaf size diminish. Calcium causes a decrease in the shoot-root ratio of *Triticum vulgare* seedlings grown in single salt nutrient solutions (Kisser, 1927). Low nitrogen, as NH_4NO_3, reduces the shoot-root ratio in water cultures of *Nicotiana rustica, Coleus* and *Tradescantia albiflora* (Mothes, 1932). In barley and corn the greater ratio of tops to roots which results from increasing nitrate in the solution may be explained on the basis of increased use of carbohydrates in the tops (Turner, T. W., 1922) through protein formation in above-ground parts. The resulting decrease in the carbohydrate supply to the roots may bring about an absolute or relative reduction of root growth (Turner, T. W., 1922). Nitrogen deficiency in *Taraxacum kok-saghyz,* however, results in a far smaller fresh or dry weight yield of roots than do deficiences of phosphorus, calcium, magnesium or potassium (Meyer, 1945). The optimum concentration of sucrose for root growth in orchids is higher than the shoot optimum, the concentration which causes the greatest root growth suppressing growth of tops when introduced through the roots (Yates and Curtis, 1949). This suggests that the shoot to root ratio is a direct response to sugar concentration in the plant and that other factors which affect the ratio act through carbohydrate metabolism (Yates and Curtis, 1949).

Removing half the leaves of beet plants causes an increase in surface of those remaining so that the total leaf area of the plant remains constant (Kokin, 1930). Decreasing the number of leaves stimulates root development, however, one-third of the normal total leaf surface being sufficient to produce the usual root weight and sugar content (Kokin, 1930). The number of rings in beet root, however, is equal to one-sixth to one-third of the normal number of foliage leaves (Aleksandrov, 1929).

OSMOTIC PRESSURE. Higher cell sap pressure, resulting from a depleted water supply, increases suction power and decreases transpiration (Fitting, 1911), but absorbing forces of the cell wall rather than osmotic pressure may govern entrance of water (Livingston, 1911*a*, *b*; Briggs and Shantz, 1912 and 1913). The vapor pressure of a one molar solution, however, is only 1.8 percent less than that of pure water. Since the vapor pressure lowering of the most concentrated leaf sap ever reported would not equal ten percent and vapor pressures of hydrated plant colloids are within two to five percent of those of pure water, neither osmotic concentration nor colloid hydration can account for drought resistance (Boon-Long, 1941). High osmotic concentration artificially induced by glucose injection, exposure to light or interruption of translocation, lowers tissue permeability to water, as tested by the plasmotic method (Boon-Long, 1941). Such decreasing of cell permeability to water by high osmotic concentration is largely responsible for the reduction in transpiration rates (Boon-Long, 1941).

Osmotic values of plant vacuoles in general range from three to 180 atmospheres (Zirkle, 1937). Relative osmotic pressures are indices of the ability of above-ground parts to compete for water (Herrick, 1933). Internal competition for water establishes consistently increasing gradients in the osmotic and suction tension values from lower to higher levels (Herrick, 1933), which accounts for internal translocation of water from lower to upper leaves during wilting (Pringsheim, 1906). In non-deciduous trees and shrubs cell sap density increases with height (Gail, 1926). Osmotic pressures in woody species are greater than in herbaceous plants (Korstian, 1924; Meyer, 1927), since herbs may not become modified physiologically for lifting water to such great heights as woody species. Osmotic pressures in leaves and internodes of *Ambrosia trifida* increase with age to a maximum, then decrease (Herrick, 1933). In *Larrea tridentata* the leaf water content normally increases with age (Runyon, 1936).

As a rule water is lower in leaves around mid-day because of the increase in photosynthate and other materials as well as from water loss, but *Larrea* and *Prosopis* may show a somewhat higher leaf moisture content by day than by night (Livingston and Brown, 1912). In *Ambrosia trifida* both osmotic and suction tension values tend to reach a daily maximum between 1:00 and 3:00 P.M. (Herrick, 1933). Reduced light causes a decrease in osmotic pressure of cell sap in both deciduous and non-deciduous trees (Gail, 1926), apparently through the effects of light on photosynthesis and transpiration.

Sun leaves of four woody prairie species have 1.1 to 11.6 percent lower water content than shade leaves and 2.1 to 5.4 atmospheres higher osmotic pressure, the difference being explained by the higher transpiration rate in sun leaves (Marsh, 1941). Dwarf red kidney bean shows a progressive depletion of starch in leaves of plants permitted to approach wilting, while there is a marked build-up of starch reserves immediately following irrigation (Wadleigh, Gauch and Davies, 1943). Osmotic pressure is affected by interconversion of carbohydrate as well as by water loss directly.

Osmotic pressures of sclerophylls from arid regions are higher than in those from forest areas (Wood, 1934), the highest cell sap densities usually occurring on the most adverse sites and in more drought resistant species (Korstian, 1924). Chemical properties of the tissue fluids depend upon environmental factors and species peculiarities, and even in a mesic environment the xerophyte often has a higher osmotic pressure than mesophytes (Maximov, 1929). The range of cell sap concentration within a species, however, is regulated by external, not internal factors (Mallery, 1935). Cell sap concentration in deep-rooted prairie plants may increase 30 atmospheres as the soil dries (Stoddart, 1935).

Osmotic pressure in different parts of the leaf is closely related to the amount of available water (Iljin, 1916). Cell sap concentration is higher along the margin of wide leaves, measuring 0.64 atmosphere on the edge of a *Verbascum lychnitis* leaf and 0.43 atmosphere in the middle; osmotic pressure in the narrow leaves of *Saxifraga aizoon* measured 0.62 atmosphere, both in the middle and along the margin (Iljin, Nazarova and Ostrovskaja, 1916). Low osmotic pressure in leaves, identical with that of roots, occurs in plants thickly surrounded by a dense stand, osmotic pressure in *Poa pratensis* under these conditions measuring 0.37 atmosphere in both the root and leaf (Iljin, 1916).

Leaf compactness, in contrast to expansion, is associated largely with high relative transpiration, thus with high osmotic pressure (Coulter, Barnes and Cowles, 1931). The varying degree of cell sap density, acting on protoplasm, appears to be the stimulus and modified structure the effect (Schimper, 1903). Palisade, however, is usually best developed adjacent to the upper leaf surface through which less water is lost.

At higher altitudes *Caltha* has a greater amount of osmotically active solutes (Montemartini, 1927). Yet investigation of 143 species shows that the ability of alpine plants to maintain themselves cannot be attributed to the high osmotic value of cell sap (Yoshi and Jimbo, 1931).

The osmotic pressure of *Larrea tridentata* root hairs known to function in absorption is ten atmospheres (Spalding, 1904). Certain older root hairs, which, from their regenerative capacity and action with plasmolyzing agents, also appear to be functional, have an osmotic pressure several times as high as young cells toward the root tip (Spalding, 1904). *Zea mays* absorbs throughout the entire root surface up to ten centimeters in length, but least is absorbed through the root cap, and less water enters through the zone of elongation than through older root parts (Hohn, 1934). As a rule, however, young root hairs have a higher equivalent (Hill, 1908). Root hairs are entirely absent from pecan (Woodroof and Woodroof, 1934) and are suberized in certain other species. The presence or absence of root hairs in *Zea mays* does not change the relative rates of absorption of the various regions in solution; yet it cannot be said that root hairs are of no importance in obtaining water from dry soil or in solute absorption (Hohn, 1934). *Triticum vulgare* seedlings grown in single salt nutrient absorb most in potassium solutions, although root hairs fail to appear, and water economy is least favorable in calcium solution in spite of well developed root systems with abundant root hairs (Kisser, 1927).

Apparently under conditions of reduced transpiration water moves by osmosis across the differentially permeable multicellular membrane formed by living root cortex (Kramer, 1932). Accumulation of water in the vessels may result in the development of a positive hydrostatic pressure or root pressure (Kramer, 1932). This pressure is responsible for exudation from excised roots (Grossenbacher, 1939; Biale, 1941). Such secretion is not retarded by an opposed pressure of 90 pounds per square inch, a force sufficient to raise water to a height of 200 feet (White, 1938).

Where water is abundant changes in the transpiration rate precede changes in absorption, water intake being determined by water loss (Kramer, 1937). Otherwise, available water limits transpiration (Spalding, 1904). The lag of absorption behind transpiration may result from resistance in living root cells between the epidermis and xylem (Kramer, 1938). The rate of water intake through a sunflower stem attached to a vacuum pump is increased 83-fold by excising the roots (Kramer, 1938). Absorption in certain other plants not attached to a vacuum pump is temporarily increased ten-fold by removing roots held at 6° C. (Kramer, 1938). Absorption then falls to a rate of 2.5 times that prior to removal of the roots, and the attached wilted tops regain turgor within five minutes (Kramer, 1938). In five- and seven-year-old pines, spruce and oaks, transpiration in the first minutes after cutting increases 20–30 percent, possibly from removal of tension in the vessels, and returns to normal intensity only after five, ten or 20 minutes (Iwanoff, 1928). *Aristolochia sipho,* with an external surface 300 times that of a spherical *Echinocactus,* loses 6000 times as much water (Schimper, 1903), apparently because of a greater resistance to flow in the cactus and because its colloids resist dehydration.

Water is more readily absorbed through dead than through living roots, shown by a vacuum pump attached to a decapitated stem (Kramer, 1933). Plants with boiled roots, however, remain fresh no longer than stems severed under water (Watson, W., 1894), transpiration drops rapidly after roots are killed, and root pressure does not exist in dead roots (Kramer, 1932). Resistance to flow in the root, greater at low temperatures because of the increased viscosity of both water and protoplasm, may be the principal reason for inability of plants to absorb sufficient water at low temperatures (Kramer, 1938).

Surface reduction in small leaves may be incidental or even illusory, but internal resistance to movement of materials, resulting from high osmotic pressure, is apparently decreased by proximity to veins and by the greater amount of palisade characteristic of xeromorphic leaves, often arranged around a central vascular bundle, diminishing the number of membranes in each radius.

LEAF DURATION. Inefficiency of the conducting system appears to be one of the internal factors which limit the life of leaves and ultimately of plants, photosynthetic activity of a leaf terminating first in the marginal and intervascular regions (Dastur, 1925). Anthocyanin formation, which ordinarily precedes leaf fall, is apparently facilitated by interrupted translocation and sugar accumulation in the leaves (Griffin, 1935). Sugars, however, appear to be transferred from deciduous leaves into the branches or tree trunk before leaf fall, as sap expressed from freshly fallen leaves is more readily frozen than that from leaves shortly before abscission (Gail, 1926).

Factors that decrease photosynthetic activity are accompanied by increased leaf duration and thus by an increase in photosynthetic area (Pease, 1917). Leaf duration on evergreen trees and shrubs is shorter in the open than in the shade and shorter on the windward than on the leeward coast, factors favoring transpiration being accompanied by decreased leaf duration and reduced transpiring surface (Pease, 1917). In the Po Valley the last leaves to fall from the poplar are those at branch ends, transpiration in poplar being chiefly stomatal, and the young leaves, having more mobile stomata, control water loss better (Montemartini, 1930). The last mulberry leaves to fall are those at the branch base, transpiration in mulberry being chiefly cuticular, the older leaves being better protected (Montemartini, 1930). In the monsoon climate of India leaf fall is more frequently an indication that the plant is about to bloom than of drought (D'Almeida and Desai, 1942). In the uniform climate of Singapore, trees of a number of deciduous species change leaves annually, many in February, others in August, apparently because of leaf senescence (Holttum, 1931).

TEMPERATURE. Temperature affects the plant indirectly as one of the factors influencing the moisture deficit (Maximov, 1929). Both respirational and transpirational losses from the leaves increase progressively with each successively higher temperature where bean, milkweed and tomato plants are held in darkness overnight at 4°, 10°, 20° and 30° C. (Hewitt and Curtis, 1948). Transpiration in *Helianthus annuus* six to eight weeks old varies little with soil temperature between 55° and 100° F., but falls rapidly below 55° F., is reduced to half at 38° F., and approaches zero at 32° F. (Clements and Martin, 1934). Small leaves and leaves arranged parallel to incident light rays may be less rapidly heated by the sun. About 50 percent of the total radiant energy of the sun is transformed in the leaf and used in vaporizing water, more rapid in sun than in shade leaves (Marsh, 1941). Under ordinary circumstances thermal emission accounts for more than 50 percent of the heat lost from leaves of *Liriodendron tulipifera* (Watson, 1934). Transpiration, higher in xeromorphic leaves, may at times prevent leaves normal to the sun from being injured, waxed apple leaves becoming 6° to 9° C. warmer than those which are transpiring (Wallace and Clum, 1938). There is no significant temperature difference, however, between waxed or blackened *Opuntia* pads and controls (Wallace and Clum, 1938). The cooling effect of transpiration is small compared with effects of light intensity, angle of incidence, convection, radiation and air currents (Clum, 1926*b*). The maximum difference observed between a transpiring leaf and air temperature in the open is 13.1° C. (Clum, 1926*a*). Leaf temperature seems to depend more on light intensity than on transpiration rate (Clum, 1926*b*). Changes in leaf temperature as great as 10° C. in one minute may be induced by natural or artificial changes in light intensity or in rates of air movement (Curtis, 1936). Conclusions of earlier workers that leaves in direct sunlight may be cooled by transpiration to below air temperature are questionable, since the methods used may give readings too high by 2° to 10° C., and leaves may be cooled 2° to 3° C. by radiation to thermocouples threaded into them (Curtis, 1936).

Increased temperature and higher relative atmospheric humidity cause leaf fall in rooted *Euphorbia pulcherrima* plants (Schnee, 1934). *Mathiola* has deeply notched leaves and no flower buds when kept at 60–70° F.; entire leaf margins and no flowers when maintained at 60–70° F. during the day and 50–60° F. during the night; and entire leaf margins and flowers when kept at 50–60° F. continuously (Post, 1936). While cooling impedes absorption and may lead to wilting in moist soil, young shoots of oats, barley and wheat continue to give off water when the soil temperature is nearly as low as 0° C. (Maximov, 1929). The root system develops better but more slowly at low temperatures (Maximov, 1929).

LIGHT QUALITY. Small leaves are exposed to a different quantity and quality of light. They are reached directly by rays containing all parts of the spectrum, not having been reflected from internal surfaces of other leaves (Thoday, 1931). Better development, expressed in stem thickness and leaf size, is obtained in the full solar spectrum than in any portion of it (Popp, 1926; Pfeiffer, 1928). Poor growth under a forest canopy, however, is attributable to light intensity rather than light quality (Shirley, 1929).

CHLOROPHYLL CONTENT. Xerophytes have a higher rate of photosynthesis in spite of a decreased total chloroplast surface (Priestley, 1929; Thoday, 1931) and possibly paler chloroplasts, as where there is excess soil salt (Weaver and Clements, 1929). The sun leaf of *Taraxacum officinale*, however, is greener than the shade leaf (Gier and Burress, 1942). The chlorophyll concentration per unit area of internal surface is greater in high light intensity in the mesophytic *Vinca rosea*, but lower in the xerophytic *Nerium oleander* under the same conditions (Turrell, 1940*a*). Reducing light intensity to 12 percent of normal sunlight increases chlorophyll and carotinoids, but increasing duration of illumination decreases total chlorophyll (Guthrie, 1932). Chlorophyll concentration increases with decreasing light until the intensity is so low as to hazard survival (Shirley, 1929). The relative chlorophyll content in individual leaves of tropical plants varies with age, being low in young, highest in mature and declining in older leaves (Dastur and Buhariwalla, 1928).

The chlorophyll concentration in corn leaf tissue is not significantly correlated with yield (Miller and Johnson, 1938), but in *Ilex sugerokii leucoclada* the ratio of assimilative activity in one-, two- and three-year-old leaves is 1.00 : 1.53 : 0.87, respectively, and assimilation is positively correlated with chlorophyll content (Hiramatu, 1939). Intercellular space is significantly greater in basal apple leaves, but apical leaves, which are more xeromorphic, seem adapted to a higher CO_2 assimilation rate per unit of leaf area, due to the greater chlorophyll content and to the exposure of a larger internal cellular area per unit of leaf surface (Cowart, 1936). The palisade of normal apple leaves contains 85 percent or more, and spongy mesophyll 15 percent or less of the total internally exposed surface of apple leaves (Pickett and Birkeland, 1942). The extent of internally exposed area in apple leaves is evidently more important than chlorophyll content as a factor affecting photosynthetic activity (Pickett and Kensworthy, 1940). In mesomorphic leaves, however, palisade cells ordinarily contain two to six times as many chloroplasts as in spongy parenchyma, and chlorophyll content is a fairly accurate criterion of photosynthetic capacity (Haberlandt, 1928).

ASSIMILATION. The greater internally exposed surface of xeromorphic leaves (Turrell, 1936) and the higher chloroplast content of palisade cells (Haberlandt, 1928) may explain in part their greater photosynthetic activity. Xerophytes produce more dry weight per unit of water used (Thoday, 1931), leaves assimilating more rapidly with low than with high moisture content (Dastur, 1925). The intensity of CO_2 assimilation in buckwheat and sunflower plants recovered from wilting is in some cases double that of unwilted controls (Tumanow, 1927). Measured in terms of CO_2 absorbed, photosynthesis is at a maximum in apple leaves whic have lost in drying 10–20 percent of their highest fresh weight (Alekseev, 1935). The greater stomatal frequency in xeromorphic leaves may facilitate the entry of carbon dioxide, accelerating photosynthesis (Thoday, 1931), though the stomatal openings are smaller, since the rate of gas diffusion does not decrease proportionately with reduction in aggregate pore area (Brown and Escombe, 1900). Closing of stomata at the beginning of wilting checks CO_2 metabolism (Maximov, 1929), but wilting is delayed in xerophytes. During the early part of the day photosynthesis may be correlated with light and later with transpiration, when water loss is closely related to the stomatal aperture, and under certain conditions photosynthesis is correlated with CO_2 concentration (Shirley, 1935). Under natural conditions low CO_2 limits assimilation and growth in wheat, flax and sugar cane (Singh and Lal, 1935).

Less leaf surface in sorghum is required to produce a bushel of grain in a dry than in a wet year, but the highest yields are obtained in seasons of abundant rainfall (Swanson, 1941). One plant, as sorghum or millet, may yield a pound of dry weight on one-third the amount of water required by another, as alfalfa (Briggs and Shantz, 1914). Drought resistance, expressed as ratio of dry substance accumulated per kilogram of water transpired, equals 1:300 in certain central European plants, including barley, oats and clover (Hellriegel, 1883).

MINERAL BALANCE. Total ash and nitrogen content are greater in plants grown in dry than in moist soils (Rippel, 1919), and desert soils, in consequence of low leaching, are high in soluble salt. Under conditions of high transpiration plants may accumulate more ash, calcium, phosphorus and potassium as well as dry weight, intake of certain ions increasing with absorption, but not necessarily in proportion, and the rate for each ion varies with the kind of plant (Freeland, 1936). The amount of mineral intake is not proportional to either transpiration or water absorption. Salt accumulates in living cells against the concentration gradient by metabolic activity reflected in aerobic respiration (Hoagland and Broyer, 1936). Maize roots absorb NO_3 and PO_4 most rapidly after sunset, that time in the 24-hour period when respiration of the root system is most intense (Potapov and Stankov, 1934). During the day absorption is considerably less than at night (Potapov and Stankov, 1934). Decreasing transpiration of barley by half through increasing humidity or concentration of the nutrient solution reduces the ash content slightly (Muenscher, 1922). Decreasing transpiration by shading reduces photosynthesis and available food, and the ash content is correspondingly reduced (Muenscher, 1922). Ash content decreases from lower to higher levels in tree leaves (Seiden, 1926).

In barley the influence of light and humidity on transpiration are less apparent where roots have a high potentiality for salt absorption (low salt, high sugar status) than in those initially higher in salt (Broyer and Hoagland, 1943). Stomata of *Zea, Pisum, Phaseolus, Nicotiana* and *Tradescantia* respond sluggishly to environmental factors as a result of a nitrogen, phosphorus or potassium deficiency, accompanied by increased water requirement, decreased fruit yield, smaller plant size and a lower shoot-root ratio (Desai, 1937). Nitrogen deficient oat leaves are more active photosynthetically than those of comparable plants supplied with nitrate (Lundegardh, 1932). A phosphorus deficiency in *Hordeum distichum nutans* var. *kenia,* however, reduces photosynthetic activity by half (Müller, 1945). Senescence in potato leaves is delayed by adding moderate amounts of potash (K_2SO_4) to the soil, suggesting that potassium ions increase the catalytic activity of protoplasm (James, 1930). Under conditions of nitrogen deficiency there is a definite migration of nitrogen from relatively mature or senescent tissues to meristematic zones (Mason and Maskell, 1934). Directly or indirectly, it is probably assimilation rather than absorption of nitrate which ordinarily limits growth (Nightingale, 1937; Davidson and Shive, 1934 and 1935).

Diminishing calcium is unfavorable to growth of sugar beets in ammonium cultures (Prianischnikov, 1928), while seedlings, as oats, barley and maize, relatively high in carbohydrate, and pumpkin, containing reserve fats, absorb ammonium rapidly from solutions containing ammonium sulfate or chloride (Prianischnikov, 1928). The pH and calcium content of the solution influence the relative absorption of ammonium and nitrate more than the stage of plant development (Prianischnikov, 1927, 1935). Root hair production is dependent upon an adequate supply of calcium (Farr, 1927; Loehwing, 1928), but calcium tends to accelerate transpiration (Chancerl, 1914; Hansteen-Cranner, 1914).

TRICHOMES

Trichomes are most abundant in xerophytes (Fig. 5), being in part responsible for the grayish aspect of desert vegetation. The shape and structure of trichomes are inherent, external factors determining only their presence or absence (Yapp, 1912). Where a single species exists as a mesophytic and xerophytic form, the latter is more hairy (Coulter, Barnes and Cowles, 1931). In certain plants drought increases the density of the hairy covering (Vesque, 1881), dryness stimulating the development of trichomes as it does of root hairs (Yapp, 1912). Hairy and smooth leaved plants of *Coreopsis lanceolata*, however, occur in the same habitat, and hairy and glabrous parts appear on the same *Polygonum amphibium* at the water's edge (Coulter, Barnes and Cowles, 1931). Pubescence is more apparent in upper leaves, which are more xeromorphic, and distribution of trichomes in partially hairy *Spiraea ulmaria* leaflets corresponds to localization of withering due to wind, marginal hairs being continuous with additional bands extending inward between main veins (Yapp, 1912). Possibly water inhibits hair development, as a damp atmosphere prevents development of thorns in *Berberis* (Lothelier, 1893; Goebel, 1898). Transpiration apparently acts as a releasing stimulus. There are no hairs in the leaf bud of *Spiraea ulmaria*, but pubescence develops and increases as leaves unfold, appearing first

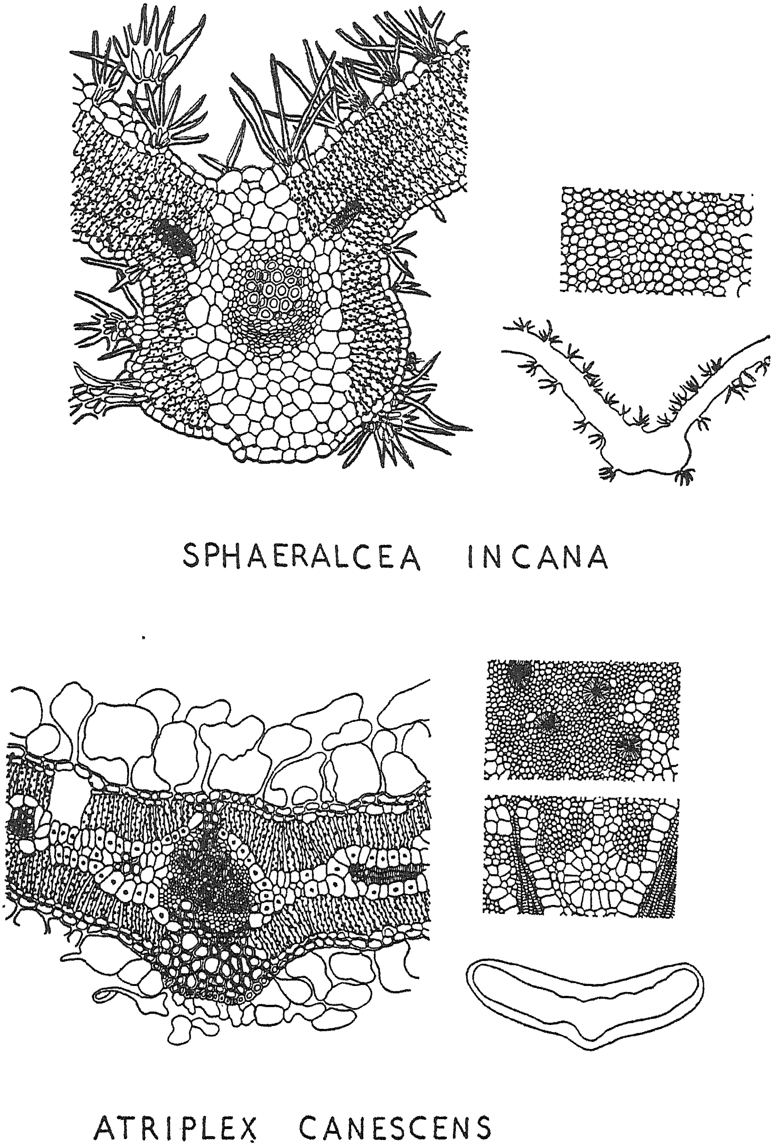


Fig. 5. Showing the stellate (above) and inflated balloon-like (below) trichomes on two xeromorphic leaf types (Shields, 1951).

along larger veins (Yapp, 1912). The initial stimulus appears to be fluctuating turgor in hair-producing cells (Yapp, 1912). *Spiraea ulmaria*, many other marsh plants and certain land plants exhibit a seasonal periodicity in hair production, a succession of glabrous, partially hairy and completely hairy leaves being followed by glabrous leaves in autumn (Yapp, 1912). Strigose hairs probably function mainly while organs are young, being denser at this time and becoming scattered as the surface increases (Coville, 1893). Hairs on unfolding leaves and growing shoots retard transpiration during windy and sunny times in spring before cuticle is fully developed (Kerner and Oliver). The trichomes on foliage of *Tussilago farfara* are effective only in wind (Brewig, 1933), stomata being exposed to suction in the wind so that gas exchange involves not only diffusion, but also the effect of moving air (Netolitzky, 1926).

Insect galls on smooth leaved species of *Vitis* are covered with a hairy coat, possibly because gall insects introduce osmotically active substances exerting an action on the leaf comparable to a xeric environment (Coulter, Barnes and Cowles, 1931). In swellings at the bases of glandular hairs of *Giardinia zeylanica* and similar cells of Vitaceae and Chenopodiaceae species potassium, associated with chlorine or carbonate anions, accumulates to the exclusion of any other cation (Lukaszewicz, 1926). Potassium salts increase osmotic pressure (Garner, McMurty, Bacon and Moss, 1923). Accumulation of KCl in these epidermal cells results from rapid transpiration (Lukaszewicz, 1926), and the resulting relatively high osmotic pressure may induce the formation of these swollen structures.

The effect of water shortage in producing outgrowths is also apparent where drought changes the branching habit of *Larrea tridentata* from one branch at a node to an opposite or whorled arrangement (Runyon, 1934).

No variation appeared in hairiness of plants subjected to increased exposure along a slope (Bright, 1928), but the hairy covering of certain *Quercus* species becomes thicker upon exposure to greater light intensity (Brenner, 1904). Being chiefly on the stomata-bearing surface, most trichomes apparently do not function in decreasing light intensity (Wiegand, 1910). Certain leaves deprived of trichomes may become 37.5 percent warmer than normal leaves (Baumert, 1907). A woolly covering which decreases light intensity and transpiration may result in a looser arrangement of chlorenchyma (Clements, E., 1905). Upon removal of felt from *Stachys lanata* transpiration increases 20–50 percent (Yapp, 1912), trichomes of this species retarding water loss, particularly in sunlight (Haberlandt, 1928). Removal of hair from mullen leaves, however, does not alter their resistance to water loss in still air and light, transpiration from internal surfaces amounting to 20 to 40 times as much as through the cuticle (Sayre, 1919). Living trichomes on fern stems markedly increase transpiration (Goebeler, 1886). Trichomes and a waxy covering have little value in reducing transpiration so long as stomata are open, but when stomata close these modifications appear to be protective (Weaver and Clements, 1929).

The known values of trichomes are few in comparison to their abundant development. Gland cells of oriental tobacco leaves contain plastids which enable them to synthesize exudate in situ and eventually to excrete it (Bentley and Wolf, 1945). In certain varieties of *Saccharum spontaneum* spiny trichomes around sunken stomata on the lower leaf surface are associated with a varietal resistance to mosaic, since wherever these trichomes occur, plant lice vectors do not have access to the stomata (Artschwager, 1940). Stomata also occur on the upper leaf surface, however, where they are not similarly protected (Artschwager, 1940).

Trichomes are inferior to a cutinous coat in reducing transpiration (Yapp, 1912), except in strong sunlight where cuticle has less protective value (Wiegand, 1910). Hairy and cutinized individuals of the same species often occur side by side, making the effect of environmental factors questionable (Wiegand, 1910). Plants in the Arctic, subjected to extreme physiological dryness, are covered with cuticle instead of trichomes (Kerner and Oliver). Cuticular transpiration, amounting to ten percent of the total in mesophytes, is practically nil in xerophytes (Weaver and Clements, 1929).

In any single species the value of trichomes probably varies according to whether they are living and transpiring or dead, forming a protective layer. Possibly trichomes are symptoms of excessive water loss rather than structures which function in minimizing it.

ABSORBING HAIRS

At high humidities certain leaves absorb rather than lose water (Thut, 1938). The leaves of more than 100 genera have been described by different investigators as capable of absorbing water (Williams, 1932). Absorption may occur through trichomes and sometimes through hydathodes which ordinarily function in secretion (Haberlandt, 1928). A cellular filament or spreading bristle with stratified cellulose walls often terminates below in a non-cutinized bulbous base with pitted walls and a dense protoplasmic layer. Adjoining subepidermal cells may be radially elongate and colorless, constituting localized water-storage tissue (Haberlandt, 1928). Since water-absorbing trichomes are usually covered by a cuticle insoluble in sulfuric acid, it is probable that their protoplasts function actively in absorption (Haberlandt, 1928). The enlarged one-celled basal portion of an absorbing hair may extend down into photosynthetic or water-storage tissue (Volkens, 1887). Water absorption by submerged leaves or parts of leaves is often sufficient to supply other leaves or leaf parts exposed to air for considerable periods of time (Williams, 1932).

During the dry season many plants on the Egypto-Arabian desert live exclusively on the dew deposited at night on their leaves (Volkens, 1887). Leaf absorption in *Chamaedaphne*, an evergreen ericad, sometimes amounts to three to four times as much as transpiration on a cold winter day, important in decreasing demands upon the root and conducting system at a time when the ground is solidly frozen (Gates, 1914). Certain mesophytes also absorb water through leaves and nodes. Most samples tested in 15 genera, 35 species and 51 horticultural varieties of fruit seedlings regained turgidity and some or all of the weight lost in wilting when immersed in water for two-hour periods (Brierley, 1935).

Absorbing hairs, however, are especially characteristic of desert plants and certain epiphytes (Spalding, 1906). Of those which are most xeric, some absorb water through trichomes and nodes, while others do not (Spalding, 1906). A series of experiments gave evidence of utilization of the foliar absorbed water (Zamfirescu, 1931). Water absorption by leaves might assume some ecological significance, but is not necessarily a useful adaptation (Haberlandt, 1928). Since in wilting plants of temperate climates dew can supply not more than five to ten percent and under ordinary conditions not more than one to two percent of the daily water expenditure, absorption by aerial organs plays no role in the general water supply of these plants (Wetzel, 1924). Moistening the leaf surface during the day increases the intensity of transpiration and may have an injurious effect.

Trichomes may also check absorption through leaves. In leathery leaves the strong cuticle offers resistance to absorption proportional to that against cuticular transpiration. Absorption is more rapid where the deficiency in turgor is greatest (Krause, 1935).

Dead spines of *Echinocactus* absorb water from a near-saturated atmosphere, but have no effect on succulence because heavy-walled tissues at the spine bases do not function in absorption (MacDougal, 1912). Water absorbed by loose bark of *Fouquieria splendens* is passed into living stem tissue, resulting in the unfolding of new leaves (Lloyd, 1912).

WATER STORAGE TISSUE

Water storage tissue may develop in the central portion of the xeromorphic leaf (Fig. 6). These enlarged living parenchyma cells have a large vacuole and a peripheral layer of cytoplasm in which a nucleus and a few chloroplasts are embedded (Haberlandt, 1928). Water tissues are the first to show evidence of wilting, their water being withdrawn by photosynthetic cells having higher osmotic pressure. As the water supply is depleted, storage cells contract and then collapse, their radial walls being thrown into folds (Fig. 7). The unthickened condition of radial walls permits this adjustment. When supplied with water the contracted cells recover rapidly, even after repeated depletion (Schimper, 1903; Westermaier, 1872). Under stress, water storage tissue bridges the gap where absorption lags behind transpiration, averting injury to chlorenchyma and preventing temporary interruption of photosynthesis (Haberlandt, 1928). Probably the same environmental influences are responsible for water storage tissue formation in xerophytes as in succulents.

The formation of water storage tissue may be related to production of organic acids, from the influence of limited gas exchange on metabolism or from excess chlorides in soil water (Delf, 1912). More recent evidence indicates, however, that formation of acid in succulents cannot be attributed to an inadequate oxygen supply to the tissues (Evans, 1932). In late stages of starvation of *Kleinia articulata,* a succulent composite, the pH rises to between eight and nine, which may mark cessation of respiration in the pith, respiratory CO_2 having previously maintained a pH not above six (Thoday and Jones, 1939). In excised barley roots containing an ample supply of available sugar, organic acids disappear when an excess of anions over cations is absorbed and form when cations are absorbed in excess (Ulrich, 1942). The respiratory quotients increase as acids disappear and decrease when acids form, but neither temperature nor oxygen affects ionic balance (Ulrich, 1942).

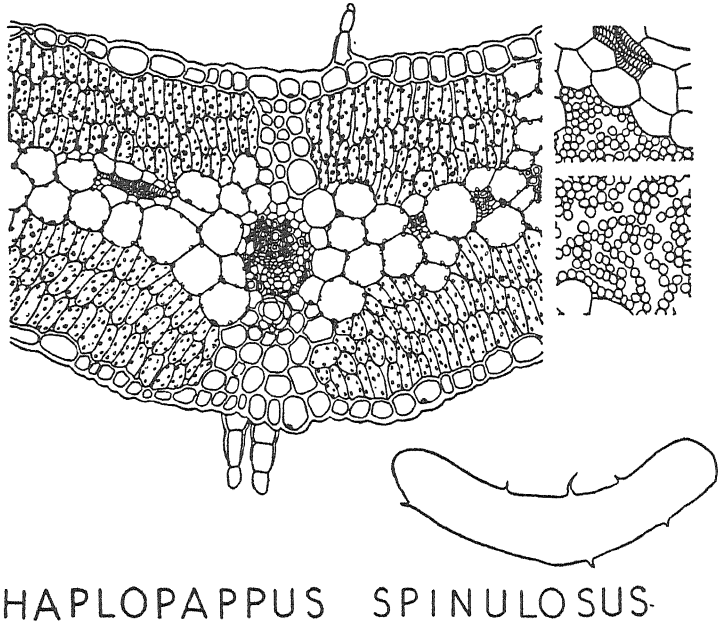


Fig. 6. Central water storage tissue in a xeromorphic leaf (Shields, 1951).

As a result of water tissue enlargement, old yellow leaves of mangrove are twice as thick as those that have just reached full size (Haberlandt, 1928). Old leaves are reservoirs upon which younger leaves draw for water (Schimper, 1903).

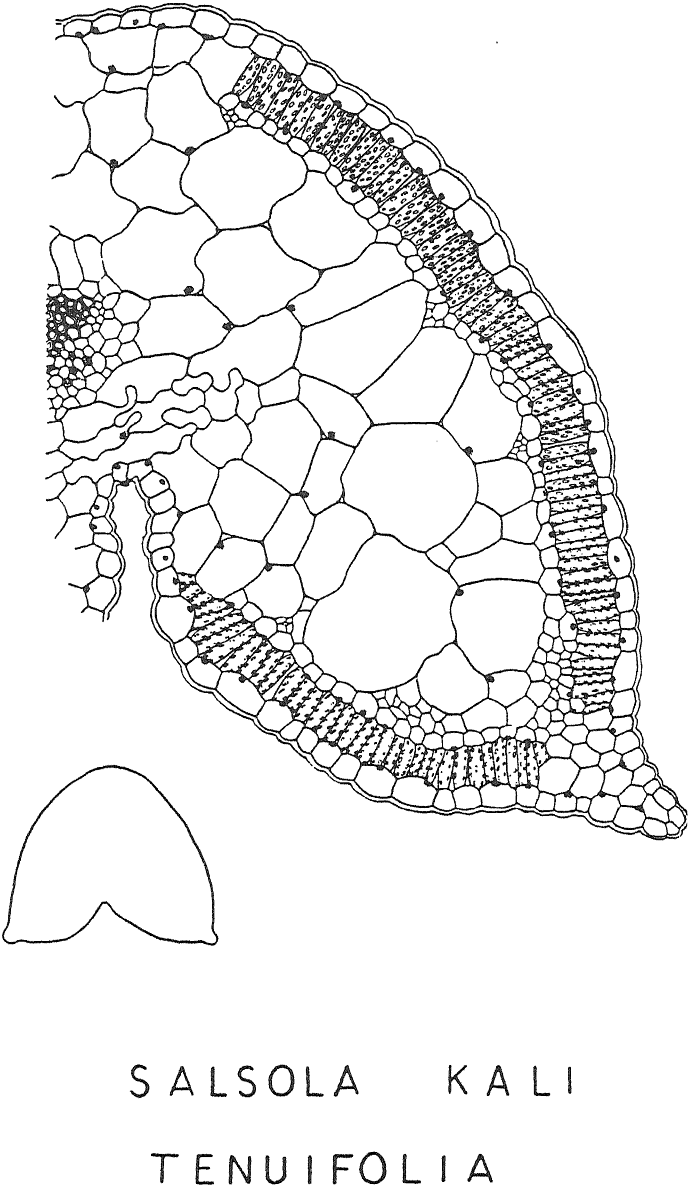


FIG. 7. Showing the collapsed condition of certain central water storage cells during wilting of a succulent-xeromorphic type of leaf (Shields, 1951).

EXPLANATION OF SURVIVAL IN XEROPHYTES

The teleological point of view assumes that the plant in a dry environment must make economical use of water. Most xerophytes are not structurally adapted for reducing transpiration (Maximov, 1931). Many xeromorphic plants peculiar to moist habitats have little drought resistance (Maximov, 1929). The effective adaptation of *Larrea tridentata* to water shortage is the ability of its protoplasm to endure desiccation without permanent injury (Ashby, 1932). The drought resistance develops as a result of a limited water supply at a critical and early stage in leaf development, in some cases before emerging from the bud. A limited water supply, high light intensity, or a nitrogen deficiency result in structural modifications, particularly a greater internally exposed area of palisade (Turrell, 1936), some of which favor excessive water loss. Under a water deficit, conversion of starch to simple carbohydrate and accumulation of photosynthate increase osmotic pressure. It is the effect of high osmotic concentration in decreasing permeability of plant cells that reduces transpiration (Boon-Long, 1941). Plants undergo changes during the day which modify transpiration, and under conditions favoring high evaporation do not respond wholly as free evaporating systems (Briggs, 1916).

Winter hardening of conifer leaves, physiologically comparable to xerophytism, may be through conversion of starch to sugars and oils (Weaver and Mogensen, 1919) and the protective action which sugars exert against precipitation of proteins (Meyer, 1928). The difference in osmotic pressure of evergreen leaves in winter and summer is not great (Meyer, 1928) and may be higher in summer (Stanfield, 1932). When osmotic concentrations are increased by lowering the temperature, hardened cabbage tissue transpires more rapidly than unhardened, hardening having made tissues more permeable (Boon-Long, 1941).

In *Vitis vinifera* and *Rheum rhaponticum* leaves the ratio of protein to soluble nitrogen increases when leaf water content falls. Diurnal fluctuations in this ratio are correlated with changes in pH, acidity increasing with protein (Newby and Pearsall, 1930). In plants abundantly supplied with nitrates, amino acids accumulate, reducing acid production (Pearsall and Ewing, 1929). Both the high amino acid content and the higher pH value allow greater swelling of protoplasmic colloids, resulting in a higher water content and reduced transpiration (Pearsall and Ewing, 1929). In general low water supply and high temperature are associated with increased polysaccharides, decreased monosaccharides and increased pentosans (Spoehr, 1919). No relation was found, however, between pentosan content of coniferous leaves and either hardiness or rate of dehydration of plant tissue (Doyle and Clinch, 1926).

Echinocactus, a succulent, in diffuse light may lose as much as 2/1000 of its weight in one day immediately after excision of its root system; the same plant six years later under equivalent conditions, except that its weight has been reduced by approximately one-third, loses not more than 1/17,000 of its weight per day (MacDougal, Long and Brown, 1913). This physiological xerophytism has been attributed to the conversion of polysaccharides into anhydrides or wall material, both transformations being induced by a depleted water supply (MacDougal and Spoehr, 1918). The factors which decrease cell permeability, however, reduce transpiration most (Boon-Long, 1941). During desiccation and starvation of cacti, respiration contributes to maintaining a water balance, 0.6 gram of water being formed for each gram of sugar burned (Spoehr, 1919).

In xerophytes there are two opposing modifications: physiologically, a high osmotic pressure, resulting in part from water loss, which produces an internal resistance to flow through decreasing cell permeability; and, morphologically, a palisade type of mesophyll, its formation induced in part by internal resistance to flow, which decreases this internal resistance to a minimum compatible with development of a stem or leaf of a given size and type.

Aridity, universally accepted as an evolutionary factor in the derivation of land plants, must also be a factor in the development of desert forms (MacDougal and Spoehr, 1918), but some of their structural characteristics appear to be incidental. Certain anatomical modifications are apparently symptoms of the changed metabolism which enables the plant to withstand water shortage, but have no survival value in themselves. Leaves from the south periphery of *Ulmus americana*, having more palisade tissue, greater structural compactness and thicker cuticle than those within the crown, lose approximately 12 times as much water (Hanson, 1917). Evaporation is one and one-half to two and one-third times as great at the south periphery as within the crown (Hanson, 1917). The correlation between xeromorphic structure and high transpiration may be explained in part by the high ratio of internal to external surface associated with the palisade type of mesophyll in xeromorphic leaves (Turrell, 1936). Stomata of xerophytes remain open longer than in mesophytes under the same conditions (Weaver and Clements, 1929). The water deficit in wilted xeromorphic leaves greatly exceeds that in wilted leaves of mesophytes (Maximov and Maximov, 1924). Drought resistance is measured by the capacity to endure water loss rather than by the rate of transpiration when the water supply is inadequate (Maximov and Maximov, 1924). Xeromorphic leaves may lose half their water content without apparent injury (Maximov and Maximov, 1924). The daily deficit in sunflower amounts to 28 percent, while that of the heliophobous plant, *Impatiens parviflora*, is 13 percent (Maximov and Maximov, 1924). Visible loss of turgescence may occur in shade mesophytes when the decrease in water content is so insignificant that it can be measured only with difficulty (Maximov and Maximov, 1924).

No direct correlation exists between drought resistance and the water requirement of plants, xeromorphic leaves being spendthrifts where water loss is concerned. Survival depends upon the ability to withstand desiccation without permanent injury (Maximov, 1931). This resistance develops as a result of purely physical conditions in the plant and soil (Emerson, 1947), and is explained on the basis of changes within the cells, such as increase in osmotic pressure, decrease in cell permeability, and modifications in the protoplasm which increase its water-holding capacity. Structural changes which may or may not further effect the conservation of water are merely the manifestations of these more fundamental physiological changes (Maximov, 1929).

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SOME MYCOLOGICAL ASPECTS OF PENICILLIN PRODUCTION[1]

D. PERLMAN[2]

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INTRODUCTION

While numerous reports and reviews have appeared during the past few years discussing various methods of administration and clinical application of the various penicillins, and while the recent summaries concerning the chemistry of the penicillins (37, 41) provide an index to the voluminous literature on the subject, the comparable summaries available of the studies on the mycological production of penicillin do not include many of the aspects of that production. During the past war distribution of information concerning penicillin chemistry and manufacture was restricted by government directive, and often the reports appearing in the literature during that period, while of general interest, had little practical application. Some of this hitherto restricted information has been reviewed by Foster (88*a*) and Raper (196, 198), together with those authors' personal experiences, and other reports concerning microbiological aspects of the production of penicillin and other antibiotics are now available. The following review will draw on some of these and other sources of information to illustrate the changes in techniques which occurred during the development of the fermentation phase of the penicillin industry during the past ten years. It is unfortunate that no coordinated effort has been made by the group of laboratories which worked cooperatively during the period of 1942–45 to publish a summary of their findings, as has been done for the chemistry of the penicillins, for even the negative results would be of interest to students of microbiology.

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[1] This review was written during December, 1949, and an effort has been made to include most of the literature appearing prior to that date. Several months later the study by Florey et al. (*73a*) became available and should be consulted for a more extensive treatment of some of the topics.

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Perhaps this has been due in part to the voluntary restrictions covering secrecy of industrial developments and practices which necessarily operate in the highly competitive chemical and pharmaceutical industries. In order to overcome this deficit, reference will be made to some of the patent literature which reflects in part some of the industrial practices. It is recognized that while this source of information is often relatively ancient history—five years have at times elapsed between application for patents and the granting of them—and frequently misleading, it has some value in indicating which practices have been tested and perhaps used in industry (105).

Recovery of the penicillin formed during the fermentation phase will not be discussed in this review, and readers are directed to the publications of Whitmore et al. (253) and others (41, 54, 32, 97) for information on this subject. This operation is fully as complicated as the fermentation phase, and, as may be seen from a flow sheet of a process formerly used by Gordon et al. (97), many steps are involved in recovering the small amounts of antibiotic from the fermented medium. While this process (Fig. 1) is perhaps more complex than those now used, it indicates that a small

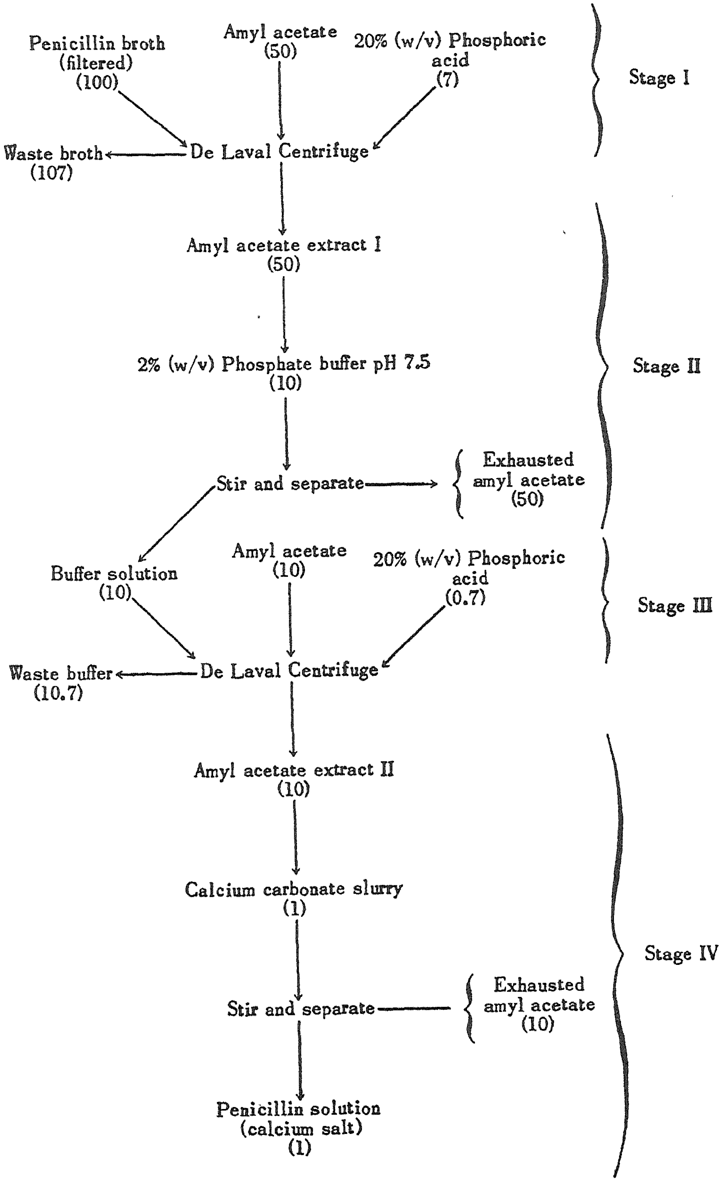

Penicillin broth (filtered) (100)
Amyl acetate (50)
20% (w/v) Phosphoric acid (7)
Stage I
Waste broth (107)
De Laval Centrifuge
Amyl acetate extract I (50)
2% (w/v) Phosphate buffer pH 7.5 (10)
Stage II
Stir and separate
Exhausted amyl acetate (50)
Buffer solution (10)
Amyl acetate (10)
20% (w/v) Phosphoric acid (0.7)
Stage III
Waste buffer (10.7)
De Laval Centrifuge
Amyl acetate extract II (10)
Calcium carbonate slurry (1)
Stage IV
Stir and separate
Exhausted amyl acetate (10)
Penicillin solution (calcium salt) (1)

FIG. 1. Recovery of penicillin from fermented media. The numbers in parentheses refer to volumes of solution in liters. (Reprinted through courtesy of Drs. T. White and J. J. Gordon (97)).

change in the type of medium employed in the fermentation phase might upset the whole recovery process by causing emulsions in the solvent extractions or changing the distribution of the antibiotic between the solvents and water or buffer phases. This aspect of the production of penicillin has often been overlooked by those studying the fermentation phase, and perhaps explains why problems in the recovery of penicillin must always be kept in mind when new media or fermentation changes are suggested.

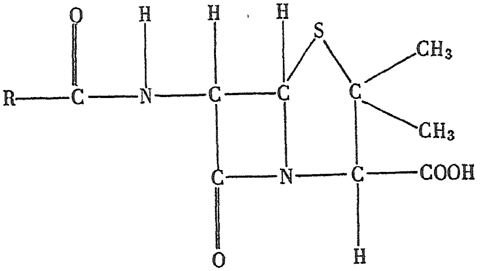
HISTORICAL BACKGROUND

Slightly more than 20 years have passed since Dr. Alexander Fleming (70) observed that colonies of a culture of *Penicillium,* first identified as *P. rubrum* but later classified as *P. notatum* (43), growing on an agar plate inhibited the growth of adjacent colonies of *Staphylococcus.* While this observation is of considerable historical importance, the founding of the large penicillin industry perhaps dates more directly from the studies of the Oxford University group which, as related by Dr. Florey (72), started its investigations in 1938. In the intervening period between Fleming's studies and the first report by the Oxford group (36), publications by Clutterbuck et al. (43) and Reid (202) indicated that study of the active bacteriostatic agents elaborated by this culture of *P. notatum* would present problems, since those agents were so unstable chemically and did not represent major metabolic products of the mold. Indeed these investigators were uncertain whether they were studying one or many factors, and it was considered possible that a protein or similar complex substance was under study (202). For a fuller description of the events leading up to and including the Oxford studies consult the authoritative report by Florey et al. (73*a*).

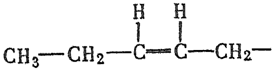
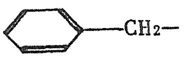
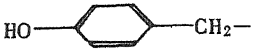
Fleming applied the name "penicillin" to the culture filtrate containing the active agent (70, 71), but this name was later restricted to the antibiotic substances present in the fermented medium which were produced during growth of the fungus. As will be discussed later in this review, several structurally related chemical entities are formed by most of the penicillin-producing cultures. These are often termed the "natural penicillins" and are usually called by trivial names, e.g., penicillin-G and penicillin-X, rather than by their chemical names, viz., benzyl penicillinate and *p*-hydroxy benzyl penicillinate. The relationships of these "natural penicillins" to each other are apparent from their formulae presented in Table I. Since many of the early studies did not indicate which of the penicillins was under investigation, the term "penicillin" will be used here to indicate the antibiotic activity as measured by inhibition of growth in *Staphylococcus aureus*. In fact it was not until after crystallization of benzyl penicillinate (penicillin-G) had been achieved in 1943 (41, 255) that definite evidence was obtained that more than one type of penicillin is produced by the mold. Many of the fungus cultures studied were found to produce an enzyme which released hydrogen peroxide and

TABLE I

FORMULAE OF SOME OF THE NATURALLY OCCURRING PENICILLINS



β- lactam structure

R	trivial names	antibiotic activity against *Staphylococcus aureus*
		units per milligram
 $CH_3—CH_2—\overset{H}{\overset{\vert}{C}}=\overset{H}{\overset{\vert}{C}}—CH_2—$	Penicillin I; Penicillin F.	1500
$CH_3—CH_2—CH_2—CH_2—CH_2—$	Gigantic acid; Dihydro penicillin F.	1500
	Penicillin II; Penicillin G.	1667
	Penicillin III; Penicillin X.	950
$CH_3—(CH_2)_5—CH_2—$	Penicillin IV; Penicillin K.	2250

whose antibiotic activity was often confused with that of penicillin. Discussion of the properties of this enzyme is outside the scope of this review, and the reader is referred to the literature for a description of this enzyme, often called "notatin", "penicillin-B" and "penatin" (52, 53, 131–133, 204), but which seems identical with the glucose aerodehydrogenase studied by Müller in 1928 (166, 167). The presence of this enzyme in filtrates from fermentations does not affect the determination of penicillin content by the agar diffusion assay method (described below) and has not complicated interpretation of data collected by this method.

The success of the preliminary studies in treatment of bacterial infections reported by the Oxford group (36, 2) indicated the need for expansion of penicillin production facilities and the study of penicillin as a potential means of treating war-incurred injuries. This need was first recognized in Great Britain and resulted in the formation of the Therapeutic Research Corporation whose membership included representatives of the Boots Pure Drug Company, Ltd., British Drug Houses, Ltd., Glaxo Laboratories, Ltd., May and Baker, Ltd., and the Wellcome Laboratories. Research progress reports were circulated among these members, and in 1942 the importance of the work caused the Ministry of Supply to organize a general commission to deal with the various aspects of the production of penicillin—the Penicillin Producers Conference. This group included at times members of the above group and representatives of Imperial Chemical Industries, Ltd. (Alkali and Pharmacetutical Divisions), Imperial College of Science, Manchester University, the National Institute for Medical Research, and several laboratories of Oxford University (41).

The difficulties of expanding production of penicillin during the war emergency in Great Britain prompted solicitation of assistance from laboratories in the United States. During the early part of 1941 investigations were started on a small scale at the laboratories of the Squibb Institute for Medical Research and Merck and Co., Inc. A visit by Drs. Florey and Heatley of the Oxford group to various laboratories in the United States during July–September, 1941, encouraged interest, and several other laboratories started to study the problems involved in the mycological production of penicillin, including those of the Fermentation Division of the Northern Regional Research Laboratory of the United States

Department of Agriculture, located in Peoria, Illinois. Here two important observations were made by Dr. A. J. Moyer and associates shortly after inauguration of the experimental program. These included the observation that addition of cornsteep liquor to the modified Czapek-Dox medium previously used resulted in increasing the production of penicillin five- to 12-fold, and that cultures of the penicillin-producing fungus would grow well and produce penicillin when cultured under submerged conditions in aerated vats (44). Somewhat later in that year, with the approval of the concerned departments of the Department of Justice, representatives of the Squibb Institute for Medical Research and Merck and Co., Inc., agreed to exchange information, and they were later joined in this "pool" by representatives of Chas. Pfizer and Co. A similar "pool" was formed subsequently by representatives of Abbott Laboratories, Inc.; Parke, Davis, and Co.; Eli Lilly and Co.; and the Upjohn Co. Several other laboratories also studied the problems associated with the production of penicillin, including those of Commercial Solvents Corp., Winthrop-Sterling Co., Schenley Laboratories, Inc., and Hoffmann-La Roche, Inc. In December, 1941, a meeting was held at which information was exchanged among those interested in the problem, and several sessions took place in the years following.

Production methods were explored during the next 18 months, and several producers started planning construction of new facilities and large-scale production units. This expansion was held in check until clinical data were available emphasizing the importance of penicillin as a chemotherapeutic agent, and, more important from a mycological production viewpoint, until studies on the chemistry of the penicillins indicated whether or not an economical method of chemical synthesis was to be expected. The studies on the chemistry of the penicillins were coordinated in 1943 by a section of the Office of Scientific Research and Development (ORSD), and, as related in the monograph (41), representatives of 19 laboratories in the United States and ten in Great Britain exchanged information periodically. The magnitude of the effort may be gauged from the observation that over 700 reports were exchanged in less than three years by these cooperating laboratories studying the chemistry of the penicillins. The requirements of the armed forces for penicillin caused production of it in May, 1943, to become one of the projects of the Office of Production Research and Development (OPRD) of the War Production Board. At that time construction of 18 production plants in the United States and two in Canada was in progress or contemplated (44, 45, 66, 67), and it appeared that a biological process would be most efficient and than an economical chemical synthesis was somewhat remote. In addition the research laboratories of several other pharmaceutical manufacturers were investigating problems involved in the production of penicillin.

The OPRD assigned the problem of increasing penicillin production to Dr. A. L. Elder who served as coordinator from October, 1943, to March, 1944, and to Dr. L. A. Monroe who assisted Dr. Elder in addition to having other assignments and succeeded him in 1944. The services of advisory groups to the OPRD were located at Pennsylvania State College where recovery methods were studied, at the Massachusetts Institute of Technology where drying techniques were investigated, at the University of Wisconsin where fermentation problems were studied (182), and at the University of Minnesota, Stanford University, and the Laboratory of Biology, Carnegie Institution of Washington, where methods of isolation of new penicillin-producing cultures were investigated (44, 67). While most of these projects were only of a few months' duration, since construction of plants and other developments proceeded rapidly, a staff of over 200 were employed at one time or another during the existence of these advisory groups, and some 80 progress reports were issued to those laboratories studying the production of penicillin. Perhaps the outstanding accomplishment of this effort of the OPRD was the selection and recognition of a new culture of *Penicillium chrysogenum,* designated as X-1612, in the latter part of 1944 which resulted in doubling the production of penicillin in a matter of a few months and more than repaid the small financial investment in these advisory groups.

The first experiments at Oxford University (2, 36) utilized the surface method of culturing the fungus. Later experiments (44, 67, 42, 66, 54) indicated that the organism would grow well and produce penicillin when cultivated in submerged culture, when grown on moistened bran, or when grown in a "vinegar" type generator. While most of the early production in the United

States (1942–1943) was the result of surface culture method, and while two plants were started using the bran process, the submerged culture method appeared to be the most economical from the standpoint of material cost, labor involved in operation, and production of antibiotic per unit of time (these methods will be discussed later in detail), and it was eventually adopted in 1944 as the method for the production of penicillin.

Once the production plants had started operation, the process development, as is usual in such cases, was somewhat restricted. The most marked improvement in production yields came as result of the use of new cultures (45, 196, 197) and the observation by Dr. A. J. Moyer and others that addition of phenacetyl derivatives to growing cultures encouraged the formation of penicillin-G. Production rapidly increased, as indicated in Table II, and distribution of penicillin for chemotherapeutic purposes, which had been restricted by a directive of the War Production Board allocating 75% to 85% of the production in 1943 and 1944 to the armed forces, was practically unlimited by the end of 1945.

TABLE II

PRODUCTION OF PENICILLIN IN THE UNITED STATES*

Year	Billion units
1943	21
1944	1,633
1945	7,052
1946	27,809
1947	41,426
1948	95,855
1949	133,229

*Drawn from data summarized in Chemicals and Drugs, a monthly publication of the U. S. Department of Commerce.

Most of the investment in construction of production plants and laboratory research in the United States was financed by the industrial companies involved. It is estimated (45) that less than one-third of the $25,000,000 spent in construction was financed through government loans. The first studies (36) at Oxford University were financed in part by grants from the Medical Research Council, the Nuffield Trust and the Rockefeller Foundation. The studies at the Northern Regional Research Laboratory were done under contract with the OSRD, and the laboratory investigations started by the OPRD were supported in part by grants from the government and the companies interested in production of penicillin. Sales of penicillin products had an estimated value of $200,000,000 in 1948, and during the past six years the price of 100,000 units of penicillin has dropped from $20 (1943) to less than $0.10 (1949), with the purity of the product distributed increasing from about 25% to 98%.

While the above summary is mainly concerned with penicillin production in the United States, principally because this information is available to the author and because many of the major production developments occurred in these laboratories, considerable effort was expended in other countries, notably Great Britain. By the end of 1945 production in Great Britain was approaching 200 billion units per month; in Australia, 20 billion units per month; and small amounts were produced in Mexico (45). The wartime secrecy order tended to delay development of large-scale production in other countries, and, although some effort was expended by the German government investigating penicillin production by the surface culture process, a successful process was not in operation by the end of the war. After hostilities were concluded production plants were started in nearly all of the European countries, as indicated by reports in the technical press, making use of the processes developed in the United States and in many cases following laboratory studies concluded under wartime conditions. There are plants in 25 countries manufacturing penicillin (*227a*), with nine in Great Britain, five in Germany, three in Italy, two in France and large installations in the Netherlands, Denmark and Sweden. Approximately 25% of the penicillin produced in the United States during 1949 was exported (value $40,000,000); this market is rapidly being reduced as the foreign plants reach maximum production. A few references in the Russian literature indicate that investigations were in progress there during the war, and information given to accredited visitors to the United States laboratories from the U.S.S.R., but unfortunately no information appears to be available concerning penicillin production there. Some small plants were started in China during the war, based on processes used in the United States, but here again information on production is lacking. Since the end of 1945 a rather sizable penicillin industry has been started in Japan, and a number of papers in the literature indicate that the processes used there resemble those utilized elesewhere.

LARGE-SCALE PRODUCTION OF PENICILLIN

A brief summary of the evolution of the methods used for penicillin production has been presented in the previous section. It can be seen that this development which took place over a several-year period involved a considerable amount of work, study and ingenuity before the methods used at present were organized and accepted. One of the factors complicating this development was that this was a new type of fermentation process, producing only traces of a relatively unstable product, a situation which had not previously confronted fermentation chemists. Also the situation was complicated by the overwhelming demand for this product, which meant that little time, effort or fermentation capacity could be easily spared for rather basic studies of the various problems involved in penicillin production by the mold.

It was under these rather unsettled conditions that methods suggested for use in producing penicillin were evaluated. In nearly all of the early (pre-1941) laboratory studies the cultures had been grown in small flasks where the mycelium formed on the surface of the liquid, the so-called surface culture method. Expansion of this method to large scale operation would necessitate considerable investment in equipment as well as a high operation cost occasioned by the large labor force required to operate such units. Modifications of this surface culture method, suggested and tested on a comparatively large scale, included growing the cultures on moistened bran or grain, and growing them in towers similar to those used in the production of vinegar. At the same time considerable thought was given to the use of the submerged culture method for growing fungi, a technique which had been successfully involved in the production of gluconic acid. This latter method had the economic advantage that investment costs would be less than for the other culture methods, that the operational costs of these units would be lower, and, as will be discussed later, that the production from a relatively small unit would probably be equal to that from a large surface culture unit (bottle plant). However, the submerged culture method had not been applied to the production of penicillin when these considerations were being made, and considerable doubt existed during the first studies that this method could be easily adapted to this fermentation.

As has been previously mentioned, this period of development extended from 1941 to 1943, and during this time several surface culture units, using bottles of the bran process, were constructed and operated along with the submerged culture units (44, 66, 54). During this interval the demand for penicillin far exceeded the supply, and the allocations for general use were quickly exhausted (44, 45, 203). A number of reports appeared in the literature suggesting the use of dressings impregnated with mycelium or periodically wetting the dressing with metabolism solution from fermentations. Apparently these methods were tried in a limited number of cases (106) which under the circumstances could not have been treated with penicillin preparations. For the most part these procedures suggested growing the fungus on a gauze pad soaked with a nutrient medium, and applying the whole to the wound. It is surprising that success was obtained with such crude preparations (106, 156) and that more untoward symptoms were not encountered. It is possible that if many of these poorly controlled treatments had failed, public faith in the potentialities of penicillin as a chemotherapeutic agent would have been shaken, as the use of these " home-made " preparations was comparatively widespread (191).

While the following discussion refers only to methods and problems encountered in the mycological phases of penicillin production, it should be kept in mind that the problems encountered in the recovery phases of the production were just as numerous as in the fermentation phase, and the recovery methods developed were fully as important to the successful operation of the production units as the fermentation procedures, if not more so (32, 54, 97, 253). Very often changes in fermentation procedures which appeared to result in increased production of the antibiotic could not be immediately introduced into production practice, for they also caused many problems in recovery of the penicillin from broth.

GENERAL CONSIDERATIONS. Before studying the various procedures used for the production of penicillin in detail, it seems advisable to consider some of the problems common to all of these methods. Some of these problems are the result of the chemical nature of penicillin, including its comparative instability in slightly acid or alkaline solution at low temperatures, its loss of antibiotic properties after exposure to comparatively mild oxidizing or reducing agents, and its instability after contact with solutions of salts of certain metallic elements (41). Such chemical instability had been rarely encountered previously, and many of the research groups studying penicillin chemistry or production methods had had little experience with such unstable compounds. While those studying the fermentation aspects of penicillin production were usually not concerned with some of these characteristics of penicillin, the ease with which production goals were met was often complicated by the efficiency of the recovery of the antibiotic, and if recovery procedures were unsatisfactory the fermentation unit had to produce more penicillin to meet the demand.

Two chemical characteristics which affected the mycological production of penicillin were instability in alkaline solution and instability to heat (16, 41). During fermentation the pH of the culture gradually rises, and if allowed to proceed too far it may reach 8.5 which may result in inactivation of much of the penicillin present. This may also occur while the fermented medium containing the penicillin is awaiting processing (134), and experience has shown that some care must be taken to prevent the fermentation from undergoing these changes. Inactivation of the penicillin formed during the fermentation by heat was a problem in the bran process, as oxidation of the carbohydrate constituents of the medium by the fungus was oftened accompanied by a rise in temperature. Temperature control in units where tanks or bottles were used was not difficult, and this factor was of limited importance. While penicillin may be produced by most cultures when incubated within the range of 18° to 31° C., best results apparently have been obtained when temperatures have been controlled between 23° and 25° C. (2, 227).

In addition to being subject to chemical inactivation, the penicillin molecule may be irreversibly changed by enzymatic activity which simultaneously results in loss of antibiotic activity. This enzyme has been called "penicillinase" (1, 81) and has been found in preparations from a large number of Gram-positive and

Gram-negative bacteria (7, 63, 64, 141, 140, 153, 239, 81), actinomycetes (249, 259), the yeasts *Debaromyces guilliermondi* and *Mycoderma valida* (259), and the fungus *Papulaspora* (259). Many of these organisms may contaminate the penicillin fermentations, since they occur commonly in air and water, and if such contamination occurs at least a portion of the penicillin present will be lost. Thus it is almost absolutely necessary to operate the fermentation units under conditions of complete asepsis, and when this has not been achieved some losses have been noted. A number of antiseptic compounds have been added to fermentations in an effort to reduce the spread or growth of the contaminating organisms without affecting the production of penicillin (126), and addition of borax was effective in some instances. While this may be added as a prophylactic measure, and it is possible to have contaminants growing in the fermentation which will not inactivate the penicillin (259), there does not appear to be any satisfactory substitute for complete asepsis in fermentation operations (217).

The above discussion illustrates the need for adequate methods for sterilizing the fermentation units. The fermentation medium has usually been sterilized by heat treatment to kill contaminating organisms (54, 97, 227) by procedures similar to those used in other branches of the fermentation industry (113). Use of high pressure steam has found favor in the sterilization of the air filters, the fermenters and other equipment. Inasmuch as these fungi are aerobic organisms, they require large amounts of air for growth, and methods for sterilization of tremendous quantities of air were developed in connection with the submerged culture process. This was successfully accomplished by filtration through cotton, glass wool or other adsorbents packed tightly in steamable jacketed units (99, 182, 203, 226), by heat treatment (223) or by combinations of these and other procedures (217), as will be mentioned below in discussing the various processes.

While a detailed discussion of the disposal of wastes resulting from the mycological operations in the production of penicillin is outside the scope of this review, this is a rather important problem. Approximately 500 grams of medium ingredients (solids) are required at present for the production of three grams of penicillin, and more than three-fourths of these solids eventually have to be disposed of as waste products. Some of these wastes, notably the mycelium, may be dried and utilized as animal or chicken feed where they act as sources of vitamins and proteins in the diet (129, 232, 258). Others may be disposed of by the treatment given to industrial sewage (129). This latter processing is complicated by the character of the material which has a high biochemical oxygen demand and imposes a severe strain on the ordinary disposal system. While there are no reports available indicating that the production units have been forced to close because of disposal problems, there is some evidence that as the selling price of penicillin fell the production cost declined at a slower rate, and in many cases waste disposal cost was an important consideration in determining whether a production unit was operating at a profit.

THE SURFACE CULTURE PROCESSES. In the experiments reported by Fleming (70) and Clutterbuck et al. (43) the cultures were grown on the surface of media in grass flasks of laboratory size. While this method is commonly used for the study of fungi in the laboratory, the shapes of the glass flasks are usually not well adapted for large-scale work, requiring much more space than is absolutely necessary, especially if conical in form; furthermore these flasks are usually rather fragile and subject to breakage after only slight jarring. The Oxford group (2) designed and used a rectangular parallelepiped-shaped porcelain-ware unit, equipped with a spout for addition and removal of media, which was placed in movable wire racks. This facilitated mechanical handling of large numbers of units during certain operations, including sterilization of media, incubation of inoculated units and harvesting of the fermented media.

While these porcelain-ware units had many advantages over Erlenmeyer or Fernbach flasks, they were not so widely used as the somewhat less desirable (from the standpoint of shape) glass milk bottles which were available and could withstand moderately rough physical treatment. Both the round and rectangular base units were used in penicillin production plants with the sizes varying from one pint to two quarts, depending on the manufacturer. Some pilot-plant studies were done with Pvitsky and Glaxo flasks (41*a*) as culture receptacles, although these flasks were not too well adapted to mechanical handling. Some of the methods used in a production unit have been described by Ainsworth et al. (3) and illustrate a few of the problems involved:

> "Pint milk bottles were charged with production medium at a ratio of 200 ml per bottle by means of an automatic bottle-washing and filling machine. After filling, the bottles were plugged by hand with non-absorbent cotton-wool, fitted with loose-fitting aluminum caps, and arranged in twelves in wire baskets to facilitate handling. The aluminum caps had been designed to permit a similar passage of air to that allowed by the cotton-wool plugs. When a closer fitting cap was tried it was found that the diminished aeration gave substantially lower titres".
>
> "The medium was very suitable for bacterial growth, and it was found necessary to plug as well as cap the bottles previous to inoculation because most contamination was found to occur during the cooling, after autoclaving. Sterilization of the medium was effected by autoclaving at 2–5 lbs. per sq. in. for 30 min. This was not entirely satisfactory in all cases and some contamination by a *Bacillus subtilis*-like organism occurred in uninoculated, plugged and capped bottles. Autoclaving for 15 min. at 15 lbs. per sq. in. resulted in reduction in contamination, but also affected the medium. After autoclaving, the baskets of hot bottles were packed on to monorail carriers, which supported the baskets in an almost horizontal position, and passed through a cooling tunnel in which the temperature of the bottles was brought down to that of the incubators by a blast of air. The carriers were then moved to the spray-gun room".
>
> "The spray-guns used to inoculate the production medium were Aerograph guns (type MP). Each gun was fitted with a cotton-wool air filter and a one-liter canister containing a filter of 50 per in. mesh wire gauze and connected to the gun by 6 ft. of rubber tubing. The nozzle of the gun was covered with a cap of cotton-wool which was tied in place and the whole gun put into a small calico bag. The canister head was covered with a square of cotton-cloth which was tied down around the canister. The gun and canister were then carefully packed into a specially constructed galvanized iron box and the whole sterilized by autoclaving for 1 hour at 15 lbs. per sq. in.".
>
> "The sterilized guns were taken to the sterile room where the canisters were filled with inoculum and from there to the spray gun room where the nipple of the air filter was attached to the air line (compressed air at 5 lbs. per sq. in.), the canister being suspended 2–3 ft. above the bottles to be inoculated. The gun was removed from the bag and the nozzle flamed. The operator removed the metal cap from the bottle to be inoculated and discarded the cotton plug. The mouth of the bottle and nozzle were flamed. Inoculum was injected into the bottle as a fine spray for eight seconds. The mouth of the bottle was again flamed and the cap replaced. It was found that 192 bottles could be inoculated from one canister containing about 900 ml of inoculum by one person in 20 min.".

> "The inoculated medium was incubated in two large incubators, each 165 ft. by 14 ft. and capable of holding four rows of 33 carriers. Harvesting was effected in a mechanical harvester in which bottles were inserted and drained of their contents for 3–4 minutes before being transferred to the input end of the bottle-washing and filling machine".

It may be seen from the above rather detailed description that operation of such a unit involved considerable organization, and even with the help of many mechanical devices, required much hand labor and was thus quite expensive to operate. Nevertheless, units such as this were in operation from 1940–1945 (182) and supplied most of the penicillin produced during the first part of the period. Previous studies on the production of gluconic acid (141), lipids, (181, 246) and citric acid (245) had shown that fungi could be cultured on the surface of media contained in large pans with surface areas of one to two yds.). These trays could be arranged as individual units or interconnected. Parker (174) has reported on some of the problems encountered in maintaining sterility when these units were used for production of penicillin:

> "Some idea of the magnitude of the problem can be gained from the picture of a factory area of five acres in which ½ acre of mycelial felt was growing in trays at any one time and over which *ca.* 30,000 cu. ft. of air passed per hour. Some 20 vessels connected by thousands of feet of pipework and hundreds of valves were used in the enclosed transfer and incubation of several thousands of gallons of medium. All this equipment was maintained in pure culture at the hands of unskilled operators rather than trained microbiologists. The fundamental principles which allowed successful operation of these units included: a) the technique used for preparation of starter cultures in the laboratory must ensure completely the exclusion of contaminants; b) the plant must be so constructed that all parts of it can be sterilized and that it encloses the culture from contamination; c) the air supplied to the plant cultures must be sterile; d) operation of the plant must be specified in such detail that relatively unskilled personnel can perform this in strict accordance with the simple sequence of actions laid down; e) every stage of the system of culture must be carefully checked by examination of samples for the presence of living microorganisms other than the organism being cultured".

"Re-use" of mycelium was possible in this plant. In this procedure, which has been resorted to in other fungal fermentations, the mycelial pad is grown as usual, and at the end of the incubation period the medium below the pad is carefully withdrawn and replaced with fresh medium. The time required for fermentation of the new medium is less than for the first batch, as the mycelium is already developed. This procedure was used by the Oxford group (2) who were able to replace the medium 14 times without reduction in penicillin production, and has also been depended upon by others (162) who applied this technique in surface culture and submerged culture operations.

Tremendous numbers of spores are needed to inoculate the bottles as described above. For laboratory purposes these may be produced by growing the cultures on surfaces of agar slants or similar semi-solid media. Among the agar media that have been used is one containing glycerol, molasses, cornsteep liquor, peptone and inorganic salts (159), a honey-peptone mixture (225), a beet-molasses agar (178) and other combinations (89, 198). While nearly all cultures tested grow well vegetatively on these media, such growth may not be accompanied by sporulation, and very often variation in the concentration of certain ingredients in the medium, e.g., sodium chloride, or change in the incubation conditions will be required to obtain large numbers of spores with certain cultures (92). In a study of factors affecting the production of spores by *Penicillium notatum* (89) it appeared that there was some association between the depth of the agar medium and number of spores produced per unit area. An increase in the concentration of metabolites above the normal level did not greatly increase the yield of spores, and some evidence was obtained which was interpreted to indicate that the production of spores was limited by accumulation of toxic substances in the medium.

One practice which received study in production units was to cultivate the mold on a finely divided organic material. Rice bran, bread crumbs, ground potatoes, oatmeal, rye, wheat and rice have been used (88*a*, 99). Some success was noted (158) with a mixture of barley perlings, red dog flour, wheat bran, middlings and oats. These substances are often badly contaminated with spore-forming bacteria, and as the heat transfer characteristics of these materials is low, it is often difficult to obtain complete asepsis by the commonly used sterilization procedures.

After these agar or organic media have been inoculated and the cultures have grown and sporulated, sterile water or mixtures of water and surface active agents are usually introduced into the sporulation vessels, and the spores shaken or scraped off the vegetative growth. The suspension formed may be used immediately to inoculate the fermentation units, or may be stored for a short period (at 5° C. or lower). It should be kept in mind that while it is possible to obtain large numbers of spores by these methods, not all of these spores will be viable (225), and apparently, as will be discussed later, not all cultures derived from single spores have the same penicillin-producing capacity or characteristics. While most of the spores remain viable for some time, common practice (92) has been to use spores which are less than one month old. Stock cultures have been prepared by lyophilization of spore suspensions, drying of spores on soil, or other techniques, and in general some degree of success has been obtained in keeping the cultures in a viable form.

In the production of acetic acid or vinegar by oxidation of alcoholic solutions by *Acetobacter* sp., an aerobic process has been used, and at one time it was thought that a similar fermentation apparatus could be utilized for production of penicillin (67). In this process for production of acetic acid (175) tall vats filled with beechwood shavings as a contact surface and a support for the fermentation are employed in series. The medium is introduced at the top of the first vat and trickles down over the shavings countercurrent to the natural convection of air stirred up by the heat of the oxidation reactions. Air for the oxidation is supplied through a perforated false bottom. The liquor leaving the bottom of the tank is introduced at the top of the next vat. A unit of this type was operated successfully on a pilot-plant scale for penicillin production without contamination (42), but this process was not widely used because of difficulties in operation, including temperature control, air sterilization and prevention of contamination. These same objections were encountered when adaptation of similar processes (31, 145) used in the production of citric acid were tried for the production of penicillin, and served to emphasize that penicillin production differs from the production of other substances by mycological processes.

A rather ingenious device (228) in which the mold is grown on surfaces of liquid below the surface of the liquid in the whole unit has been described. Air is introduced into the unit and trapped in pockets below the surface of the liquid. The fungus forms on the liquid-air interface and grows. The liquid is then circulated through the unit, and as penicillin is produced the liquid is harvested and fresh medium is added. This unit was tried on a laboratory scale and worked successfully, but has obvious limitations which eliminate it as a large-scale production process.

In one of the processes for the production of amylases by fungi, the organisms have been grown on the surface of bran (238). When aerated vigorously the cultures grow rapidly, and a number of strains of *P. notatum* have been found to grow well and produce penicillin under these conditions (220). Other grains, including moistened oats, have also been used (148). In one modification of the process the bran mixture was inoculated with the mold and spread in thin layers on trays over which sterile air was passed (54). The growth of the mold was allowed to proceed for the desired period, and then the penicillin present was leached and pressed from the mixture of bran and mycelium. Under these fermentation conditions contaminations were a serious problem, and also it was difficult to prevent local overheating. These difficulties were overcome in part by placing the bran medium in long horizontal drums which were provided with baffles, a means of rotation and cooling jackets. After sterilization the contents were cooled and inoculated. Fermentation proceeded during rotation of the drums, and oxygen was supplied by introducing sterile air at the trunions. Even with these modifications contamination was still a serious problem, and eventually the process was abandoned in favor of the deep-tank process.

THE SUBMERGED CULTURE PROCESS. During the past 15 years considerable interest has been evident in the growth of fungi in submerged culture, e.g., the organism is grown in media which are aerated and agitated so that the mycelium spreads throughout the liquid. Among the first to use this technique of growing fungi for laboratory studies were Kluyver and Perquin (115) who grew their cultures in shaken Erlenmeyer flasks. Shortly after the results of their experiments became known this technique or modifications of it (growing cultures in aerated bottles or aerated revolving drums) (102, 241) were used in studying the production of gluconic acid by *Penicillium chrysogenum* (157) and *Aspergillus niger* (248). These earlier studies suggested to investigators at the Northern Regional Research Laboratories (163) that it might be possible to produce penicillin by growing the cultures in this manner, and investigations were in progress with cultures of *P. notatum* in August 1941 and later with the related *P. chrysogenum*.

In the laboratory investigations the equipment used included shaken Erlenmeyer flasks, revolving drum fermenters, small aerated vats and stirred jar fermenters. (Pictures of several types of shakers and stirred jar fermenters are found in citations 74, 182, 203*a*). Many of these pieces of equipment were fabricated by those working on the fermentation problems or by machine shops in the vicinity of the laboratories, and it is not surprising that some variation in design and operation occurred. The speed of the reciprocating shakers varied among the different laboratories from 80 to 250 cycles per minute, and that of rotary shakers from 125 to 375 revolutions per minute with an amplitude of one-half to four inches. In all cases the object of the shaking was to increase the amount of dissolved air in the liquid, and this shaking often resulted in doubling the oxygen content (224). When grown under these agitated conditions the fungus assumes many morphological shapes (30, 60*a*), most often resembling a suspension of paper pulp (60*a*).

These shaken flask fermentations permitted evaluation of a number of aspects of the fermentation, including the effect of medium composition on penicillin production, comparison of the penicillin-producing ability of cultures, and the testing of certain antifoam solutions and construction materials for toxicity effect on penicillin production (226, 227). However, the method is essentially a laboratory method, and the conditions inside the flasks certainly do not resemble physically those found in aerated tanks used in production units. While small flasks containing sintered glass aerators were perhaps more like these tank units in design, they could not be used in the laboratory, since the fungus grew through the sintered glass aerators of the carborundum stones and resulted in complete clogging. This led to use of aluminum tubing which was perforated with small holes (1/64 inch) and eventually to stainless steel units which were agitated and aerated in a manner resembling that which had been developed for use in production plants (23, 26–28, 135, 182, 203*a*, 74). These stirred jar units made possible a limited study of the effects of aeration and agitation rates on mold growth and penicillin production (23, 26, 28), and penicillin production in these units often approached or surpassed that obtained in larger units. In recent studies a laboratory version of the Waldhof fermenter (25, 206, 207) has been tried, and it may indicate possible improvements of existing designs in production units. Some study has apparently indicated that recirculation units may be incorporated in fermenters for production of penicillin (65).

While operation of these stirred jar units is comparatively expensive from the standpoint of time involved and information gained from the study of experimental variables as compared with the shaken flask units, they are less expensive and more adaptable to changes than larger tank units. Most factors in the design of

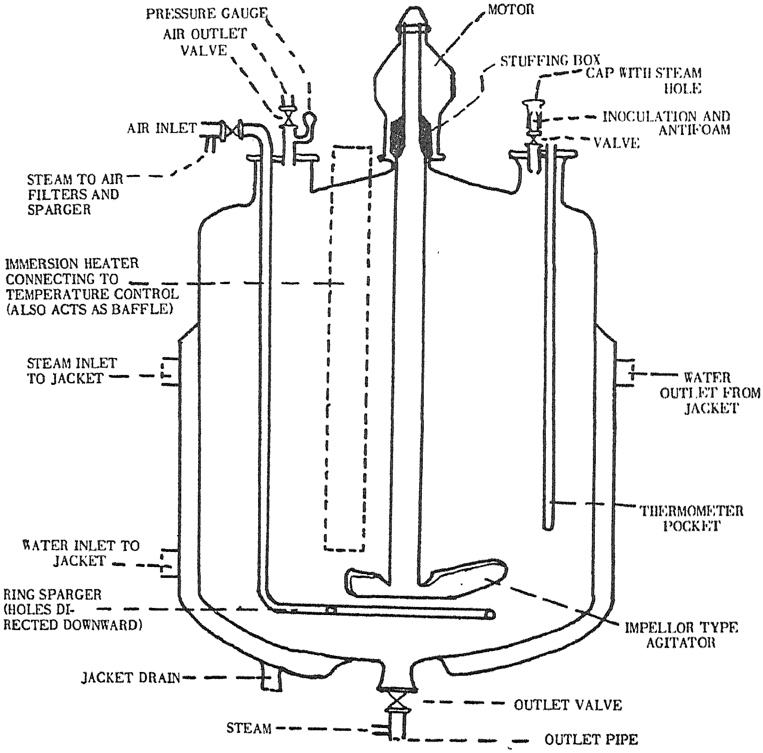


FIG. 2. Diagrammatic vertical section of pilot-plant apparatus for penicillin production. (Adapted from Gordon, *et al.* (97), reprinted with permission of Dr. T. White).

these large-scale production units have not been revealed, and apparently considerable variation exists in equipment design and operation with many types of agitators and aerating devices, metals and alloys finding some advocates among the different manufacturers. It is not surprising to hear that conditions which one manufacturer finds satisfactory will not work for another. Some of the variations may be seen in pictures of production units (32, 97, 45, 172, 185), but these reveal little of the details or operational procedures. The essential details of a pilot-plant unit are in Fig. 2, and include an air sparger, a water jacket or coils for temperature control, an agitator, sampling lines, antifoam addition mechanism, inoculation lines, transfer lines to other fermenters, cookers and recovery units similar to those in other fermentation equipment (113). Another pilot-plant unit has been described by Stefaniak et al. (226). Operating details for these pilot-plant units have been provided in the respective publications (97, 226, 74*a*, 73*a*, 190*a*). Apparently most of these units have been operated on a single-batch basis where the medium is fermented and harvested as a unit. However, some interest has been shown in operation of a continuous fermenter where medium is constantly added and withdrawn (215).

Sterilization of these units has introduced many new problems, and the complexicity of this operation may be judged by Parker's remarks (174):

> " In designing and constructing the penicillin plant we have followed a number of well defined principles which were established very early in our experience of large-scale working. These principles are as follows:
>
> a) No direct connection should be made permanently between non-sterile and sterile parts of the system.
>
> b) Welded constructions should be used where possible and convenient.
>
> c) Where joints have to be used, these should be of a high quality finish, employing rubber as the sealing material.
>
> d) The type of valve used on sterile process and service lines should be carefully selected so that it can be easily sterilized and serviced when necessary (it should be capable of maintaining pure culture conditions for the duration of the fermentation cycle).
>
> e) After sterilization, all parts of the system which are to be kept sterile should be kept under a positive pressure, either with sterile air or sterile liquor, during the cooling period and the fermentation period.

f) Each part of the system should be capable of independent sterilization without interfering with the operation of the rest of the plant.

g) Isolated danger spots which are difficult to sterilize at the start of a batch, should be provided with pressure stem connections for continuous or intermittant use".

Application of these principles usually will insure uncontaminated fermentations. A number of methods used for the sterilization of the air necessary in the fermentations have included filtration (97, 217) and heat (223).

Preparation of inoculum for submerged culture fermentations may follow the procedures of the surface culture method, namely, growing the cultures on a medium which results in profuse sporulation and inoculating the fermentation medium with a suspension of these spores. This may be done stepwise (99) : a loop of spore suspension is taken from a master stock culture kept on soil and transferred to an agar slant and incubated for five days at 25° (several types of media have been used) ; five ml. of distilled water are then added and the tube is agitated to remove the spores. The spore suspension so obtained is used to inoculate one 20-oz. Roux bottle, three plates of modified Czapek's agar and two rye grain bottles. The plates of Czapek's agar permit checking the suspension for the presence of contaminants, and they and the Roux bottle also serve to check the culture for the presence of morphological variants. Changes accompanying morphological variation may also affect penicillin production, as has been noted in at least one plant (99), and explained why penicillin production dropped markedly.

The two rye grain flasks are used as sources of spores for inoculation of a ten-liter aspirator bottle (99). The spores germinate in this aerated bottle, and the vegetative growth may then be transferred to inoculate large volumes of medium contained in fermenters. In some cases under extreme circumstances rather large fermenters (ca. 10,000 gallons capacity) have been inoculated directly with grain spore suspensions. This eventually resulted in good fermentations, but considerable time was taken while the culture was growing, and very expensive equipment was not being used for penicillin production during this time. The use of a number of intermediate vegetative stages provides a different medium for growth of the inoculum than is used for the growth of the fermentation. The reports at hand (92, 226, 88*a*) indicate that most *Penicillium* cultures grow well on media containing glucose, sucrose, starch and dextrin but that penicillin production is low on such media. On the other hand, as will be discussed later, less growth and more penicillin production are obtained on media containing lactose, and apparently these media containing glucose or glucose derivatives are used in the inoculum stages. However, one report (179) indicates that there must be some caution in selection of the inoculum medium, and results as summarized in Table III may occur if care is not exercised in choice of medium. The culture may be grown through several vegetative stages with increase in volume from ten to 1000 gallons without further treatment, or it may be homogenized at the first stage to give it a faster start (211, 240).

In the above description the cultures were grown to the sporulation stage on semi-solid or solid media. Cultures have also been observed to form spores when growing submerged in several types of media (8, 94, 183). Spore counts of 5×10^8 viable spores per ml. have been reproducibly obtained on an aqueous medium containing glycerol, molasses, sodium cloride, peptone, magnesium sulfate, iron tartrate, copper sulfate and potassium phosphate. These spores are said to be just as satisfactory for penicillin production as those obtained by culturing the fungus on solid media.

As each pilot-plant unit and large-scale fermentation unit apparently has its own peculiarities with regard to optimum rates of aeration and agitation, extended discussion of these items is not advisable here. In addition there is evidence that different cultures require different aeration rates (23, 226), and it seems that each fermentation unit must be studied by almost trial and error procedures to determine the optimum aeration and agitation rates best suited for the cultures (255*a*). Some idea of the effectiveness of the aeration may be determined by measurement of the carbon dioxide content of the exhaust gas (120, 221). In most studies where an aeration rate of one volume of air per volume of medium per minute has been found optimal, as measured

TABLE III

EFFECT OF INOCULUM MEDIUM COMPOSITION ON PENICILLIN PRODUCTION

Inoculum medium carbohydrate*	Fermentation medium**	Maximum penicillin production units/ml
none	Lactose-cottonseed meal	910
	Lactose-peanut oil meal	765
	Mannitol-peanut oil meal	765
Glucose	Lactose-cottonseed meal	850
	Lactose-peanut oil meal	735
	Mannitol-peanut oil meal	800
Lactose	Lactose-cottonseed meal	960
	Lactose-peanut oil meal	695
	Mannitol-peanut oil meal	700
Mannitol	Mannitol-peanut oil meal	725
	Lactose-cottonseed meal	835
	Lactose-peanut oil meal	760
Starch	Lactose-cottonseed meal	950
Inulin	Lactose-cottonseed meal	1055
Sorbose	Lactose-peanut oil meal	880
	Lactose-Cottonseed meal	825
Xylose	Lactose-peanut oil meal	660
	Mannitol-peanut oil meal	860

*Inoculum medium composition (grams per liter): carbohydrate, 20 g.; nitrogenous material (as in fermentation medium), 20 g.; tap water q.s. 1 liter.

**Fermentation medium composition (grams per liter): lactose, 30 g., or mannitol, 30 g., as indicated; cottonseed meal, 40 g., or peanut oil meal, 40 g., as indicated; calcium carbonate, 10 g.; soybean oil, 3.2 ml; tap water to 1 liter.

Inoculum: 5% of 48 hour vegetative growth of *Penicillium chrysogenum* Wis. Q176 grown on the indicated medium.

Abstracted from Perlman (179).

by penicillin yields, the carbon dioxide content of the exhaust gas has not exceeded 1.5%, indicating that nearly 90% of the oxygen supplied has not been utilized by the mold for oxidation of the carbohydrate. In certain studies (23) a straight line relationship has been postulated for the log of the maximum penicillin titer and the log of the respiration rate, but this relationship has not been apparent in studies in other laboratories and may vary considerably with the type of aeration equipment.

During the vigorous aeration and agitation of these fermentations large quantities of foam are formed, and, unless dispersed, considerable quantities of the contents of the fermentation vessels may froth out the air escape line. A number of anti-foam materials have been tested for ability to break this slightly acid foam, and of those examined (97, 139, 226, 227, 190*a*), vegetable or animal oils, namely, corn oil (32) or lard oil (226, 227), often containing other surface active ingredients (119, 25, 140,), have found widespread use. These are apparently non-toxic (227) to the fungus and in some cases appear to promote penicillin production, perhaps by increasing aeration efficiency (190*a*, 224) rather than reducing the destruction of the penicillin (138), or by supplying unsaturated fatty acids which may be required for rapid growth of the mold and for penicillin production (139, 19*a*).

ANALYSIS OF FERMENTATION SAMPLES FOR PENICILLIN CONTENT

The methods of the determining penicillin content of samples taken from fermentations have been of considerable importance in the mycological production of penicillin. A short consideration of these methods is included here to afford a perspective of the work that has been done, and how it has affected development of the penicillin industry. The various chemical and microbiological methods in operation prior to 1946 have been extensively reviewed in the monograph on the chemistry of the penicillins (41) and will not be developed here in detail.

Fleming (70) used an agar plate method in which a dilution of the penicillin-containing solution was added to part of the agar in the Petri dish, and the surface of the agar was streaked with susceptible microorganisms. The highest dilution at which inhibition of the test bacteria was noted was taken as indication of the potency of the preparation. Variations of this agar dilution method have been widely used since in the study of antibiotic substances (244). In the early studies at Oxford University (2) a modification of this method was devised by Dr. N. G. Heatley (101) and has since been adopted as the standard procedure. In this modification small cylinders were placed on the surface of an agar layer previously inoculated with *Staphylococcus aureus*, and solutions containing penicillin were placed in these cylinders and allowed to diffuse through the agar. Zones of inhibition of the

S. aureus growth caused by the antibiotic were measured after incubation of the plates for 14 to 18 hours. The diameter of the zone was found to be practically a logarithmic function of the concentration of antibiotic present, and comparison with size of zones obtained in the same period with standard preparations made it possible to estimate the potency of unknown solutions. This "cup-test" method, as it has been called, has been studied in detail, and many of the factors affecting the procedure have been investigated, including the effect of the pH of the samples, presence of ionized salts in the sample, composition of the agar medium (41, 101, 78, 80, 212) and inherent errors of the method (29). The difficulties in standardization have been well illustrated in the comparative studies participated in by many industrial laboratories (130), which indicated very wide variations in the analysis of impure samples.

A number of modifications of the method devised by Heatley (2, 101) have been tried at various times. These include small discs of blotting paper instead of the cylinders (41, 216, 241), and use of holes in the agar cut with an implement, e. g., cork borer, as reservoirs for the penicillin solution (29). Large dishes on which as many as 90 cylinders were placed have been substituted for the relatively small Petri dishes whose capacity is five or six cylinders (8, 29, 41), and a large number of mechanical devices have been assembled to increase the number of assays that can be done by a limited number of operators. A few of these mechanical devices have included those for quickly adding agar to plates (214), devices for placing the cylinders on the agar (40, 201) and devices for measuring the size of the zones of inhibition (171).

A large number of alternative methods to that of Heatley have been proposed at various times. In several of these inhibition of the test organism in broth culture after addition of the antibiotic solution has been measured. This may be done by turbidimetry (41, 75, 77, 12, 146) or indirectly by measurement of some other property of the culture besides growth (98). However, the presence of glucose-aerodehydrogenase (notatin) (52, 53, 131, 132, 133, 204) in the samples may affect the growth of the test organism in liquid culture if glucose is present in the medium. This enzyme does not diffuse rapidly through agar and has no appreciable effect on the determination of penicillin by the cup-test method. The presence of other substances in the samples to be assayed, e. g., fatty acids, will also affect the turbidimetric test (41), and for practical purposes the cup-test has been more widespread than any other method.

Substitution of other organisms for the cultures of *Staphylococcus aureus* and *S. albus* used by Heatley and others has been tried without much success (41, 77). When this substitution was first made the results with one test organism varied greatly from those achieved with other organisms. This was later explained by the observations that the *Penicillium* cultures under study were producing more than one type of penicillin at one time (41), and the bacteria reacted differently to the different types present in the samples. For example, penicillin–K is much more active on a weight basis than penicillin–X when *S. aureus* is the assay organism, as indicated in Table I, while the relationship is reversed when *Bacillus subtilis* is the assay organism (41). Thus, while a spore-forming organism is much easier to use as an assay organism than *S. aureus* (216, 76), it is often difficult to interpret the results (213), and since penicillin has been defined in terms of the inhibition of *S. aureus* (41), it is probably better to use the latter organism as the assay organism.

This variation in response of different organisms to the different penicillins has been relied upon as a method of estimating the proportions of the different penicillins in the samples (41, 104, 213). These methods are subject to many errors, especially if the number of penicillins in the samples under study is not known. It has also been shown that the effects of the penicillins on the test organisms are not additive (60), which limits the use of these microbiological differential methods to qualitative instead of quantitative purposes.

Other methods for determination of the various penicillins in mixtures have combined a physical separation with the microbiological assay. The mixtures have been separated by countercurrent extraction (56, 57), aqueous chromatography (235, 19*a*) or paper-strip chromatography (69, 96, 123, 124, 254), and the fractions collected have been assayed microbiologically. Study of these procedures has indicated that very small quantities of penicillin are required, about one-half unit, for complete analysis, and a rather accurate quantitative as well as qualitative estimation may be made of all the penicillins present. However, the procedures are time-consuming, and periods of 24 to 48 hours are required before the results of the analyses are available.

Several chemical methods have been proposed for the quantitative determination of penicillins, but many of these are applicable only to purified samples. Total penicillin may be determined (74) or specific analyses may be made for penicillin–G (21). Inasmuch as the main commercial interest at present is in the production of penicillin-G, these chemical methods may be quite satisfactory, and knowledge of the identity and quantities of the other penicillins in the samples may not be desired or necessary.

The above discussion has been included in this review because it is felt that one of the most important aspects of penicillin production is an understanding of the importance of the part played by microbiological assays. If the penicillin content of samples from fermentations are not accurately determined, the conclusions regarding the value of certain media, choice of cultures or certain operations may not be easily made, and much effort will be (as has been) wasted in repeating critical experiments and pondering over apparent intangibles. As can be seen from the above discussion, a number of methods have been used over the past few years, and these have changed with time as a result of better understanding of the constitution of the penicillins. Thus interpretations of the data collected in some of the early studies (pre-1945) and published in papers appearing during that period should be different than originally proposed. In the following discussion an effort will be made to reevaluate these studies in the light of the more recent developments.

SELECTION OF CULTURES FOR PENICILLIN PRODUCTION

HISTORICAL DEVELOPMENT. All of the early studies (pre-1941) on penicillin production were made with the original Fleming culture, and during that period the hypothesis seems to have developed that this particular culture was unique in its ability to produce penicillin. This theory did not appear reasonable in view of experience with other mold fermentations, including those producing citric acid, gluconic acid, lactic acid, sterols and oxalic acid, where considerable variation was noted in the production of these products by different strains of the same genus. The Fleming culture also appeared to throw off variants which were less desirable as penicillin producers (202) and perhaps accounted for some of the low yields noted (2).

Later studies (1942) at the Squibb Institute for Medical Research showed that a monospore isolate from the Fleming culture produced significantly more penicillin than the parent culture in repeated experiments. Further application of selection technique resulted in the isolation at the Northern Regional Research Laboratory of a rather stable culture from this Squibb culture which was designated as NRRL 1249.B21 and found use in the surface culture process. Most of the penicillin prepared for the earliest chemical and therapeutic studies was produced by this culture. As might be expected from its background as a natural variant, it was likely to "throw off" variants. A study of these variants (79, 250, 192) indicated that many produced less penicillin than the parent culture. Morphological variation was noted in two directions, namely, increased sporulation and pigment production, suggestive of the original Fleming culture; and reduced exudate formation and decreased sporulation in the direction of sterile colonies. One study indicated that mass spore transfers did not maintain good production of penicillin by this strain, the fungus apparently undergoing rapid natural variation with the production of mutants which usually sporulate both more rapidly and more heavily than the parent types (250). Low-yielding mutants derived from this high-yielding strain were much more stable than the parent, and attempts to build up penicillin yield of one of these low-yielding mutants by continuous selection were unsuccessful.

At the same time that studies were in progress with strains derived from the Fleming culture, other investigations were started, studying the production of penicillin by other cultures of Penicillia. A group of cultures belonging to the *Penicillium notatum-chrysogenum* group were tested for penicillin production when grown in surface and submerged culture, and nearly all of the 35 examined were found to possess some antibiotic activity (159). Consideration of this rather extensive study as well as others (85, 243, 219) on cultures of the *P. notatum-chrysogenum* group suggested that *a*) the capacity to produce penicillin as a metabolic product represented a group rather than a strain characteristic, *b*) different members of the group varied greatly in their capacity to produce penicillin, and *c*) particular strains were especially suited for certain production methods.

One of the *P. notatum* cultures, designated as NRRL 832, was widely used for penicillin production by the submerged culture method during the period 1942-1944. Attempts to isolate higher-yielding substrains from this culture by various techniques failed to produce any markedly superior penicillin-producing cultures (110) but did reveal some striking cultural (192, 221) and physiological variations (135).

A survey of other strains of *P. chrysogenum* for penicillin-producing ability when grown under submerged conditions was made in a number of laboratories, including the Northern Regional Research Laboratory (193, 195) and the Department of Plant Pathology, University of Minnesota (44, 182). A large number of cultures belonging to this genus were isolated and found to produce penicillin under these conditions. One of those isolated from a molded melon and designated as NRRL 1951 produced approximately as much penicillin in the first studies as did NRRL 832, and substrains from this culture were found to yield nearly twice as much penicillin as the parent. One of these, NRRL 1951.B25, was selected for further study, and although continued investigation of natural variants of this culture failed to reveal any subculture capable of producing higher penicillin yields than the parent stock, the widest variety of cultural types was separated out (45, 193).

This culture, NRRL 1951. B25, found wide use in the industry during the period 1944-45, and at least part of the increase in penicillin production during this period (Table II) was due to the change in cultures. When spores of this culture were subjected to x-ray and then the survivors studied for penicillin production, some increase in penicillin yield was noted with one culture designated as X-1612 (58, 121). This culture produced approximately twice as much penicillin as its parent on a cornsteep-lactose medium not supplemented with a penicillin-G precursor (121, 227, 92), and found immediate use in the industry. At this time interest centered on producing penicillin-G, since chemotherapeutic studies had suggested that this type of penicillin is as desirable as any and perhaps more effective than most as well as being more stable in aqueous solutions (17, 41). Study of the types of penicillin produced by culture X-1612 indicated that large amounts of penicillin-K were being produced on certain media, although the analytical methods used at that time were admittedly somewhat inaccurate. Supplementation of the media with a penicillin-G precursor resulted in an increase in the penicillin-G content of the penicillin formed and usually an increase in the total amount of penicillin produced (85, 190, 104).

While these studies with X-1612 were underway, investigators at the University of Wisconsin were studying the possibilities of increasing penicillin production by substrains of this culture through treatment of the parent strain with ultra-violet light. During the spring of 1945 a culture was isolated which produced considerably more penicillin than did the parent (6, 85), and when tested on various media it was found to be a consistently higher penicillin producer than the X-1612 culture. This culture, Wis. Q_{176} as it is now known, when grown on synthetic media produces very small amounts of penicillin-G and large amounts of the aliphatic type penicillins (123, 254). When grown on media supplemented with the phenylacetyl type compounds it produces large amounts of penicillin-G and small amounts of the aliphatic type penicillins (123, 218, 7). It has been widely used industrially since its first tests in October, 1945 (85). Efforts to induce mutations in this culture have been in progress during the past few years, and some success has been noted, with several cultures reported to produce several times as much penicillin as this relatively famous culture (7, 25, 88, 200).

The above discussion has indicated very briefly what success was noted in increasing the production of penicillin by culture selection. A few of the cultures used industrially and some ot their characteristics are summarized in Table IV which may aid in making a comparison of the ability of these cultures to produce penicillin when grown in submerged culture. Indeed perhaps the most marked increases in penicillin production were noted after introduction of new cultures, and at times it seemed that perhaps the easiest way to increase penicillin production was to find a new culture. This idea found favor with certain of those operating some of the penicillin plants, but placed a tremendous

TABLE IV

CULTURES USED FOR THE PRODUCTION OF PENICILLIN

Designation of cultures	Source (parent culture)	Method of Selection	Penicillin production* on cornsteep-lactose medium: Not supplemented with penicillin G precursor	Penicillin production* on cornsteep-lactose medium: Supplemented with penicillin G precursor	Ref.
			units/ml	units/ml	
NRRL 1249.B21	Squibb 4	natural variant	143	194	161
NRRL 832	T. C. B-69		95	141	161
NRRL 1951	...	new isolate	100	...	195
NRRL 1951.B25	NRRL 1951	natural variant	175	250	195
X-1612	NRRL 1951.B25	x-ray	200	350	85
Wis. Q_{176}	X-1612	U-V ray	650	1,300	85
...	Wis. Q_{176}	U-V ray	1,100	3,050	88

*In laboratory shaken flask tests for all cultures except NRRL 1249. B21 where surface culture process was used.

responsibility on the mycologist working with the cultures. Literally thousands of cultures were tested before the isolation of Wis. Q_{176}, and it is probable that many thousands more were tested before new ones were developed from that parent. While this may be the first time that mycologists were accepted as important investigators in the fermentation industry, they had to work to produce their contribution, and often the odds against which they operated are not appreciated.

The above discussion has been concerned with the production of penicillin-G and with isolation of cultures particularly suited to the production of this type of penicillin. At one time there was a demand for penicillin-X, and a study was started at the Northern Regional Research Laboratory for production of this substance. Investigators at that laboratory were able to isolate a culture which produced large amounts of penicillin-X when grown under submerged conditions (194). If there is a demand for this type in the future perhaps this and other cultures derived from it will be used.

METHODS OF SELECTING HIGH-PRODUCING VARIANTS FROM PENICILLIN-PRODUCING CULTURES. As has been mentioned earlier in this discussion, studies with the Fleming culture suggested that some improvement in penicillin production could be obtained by study of the penicillin-producing ability of monospore isolates from this culture (192, 79, 197). These and other studies indicated that considerable variation was to be expected between different cultures selected in this manner (209, 209*a*), and, while one study suggested that stability of penicillin-producing ability was to be found when spores were used as sources of inoculum (79), another concluded that at least with culture NRRL 1249.B21 a vegetative transfer was to be desired if natural variation and consequent change (reduction in this case) in penicillin production was to be avoided (250). In studies with cultures used in submerged processes some improvement was noted in penicillin production when the production of a number of natural variants of culture NRRL 1951 were tested, and one of these, identified as NRRL 1951.B25, consistently produced more penicillin than the parent culture (192, 197). This approach has been used in trying to isolate improved cultures from Wisconsin Q_{176}, but no significant increases have been reported (200). Perhaps this is an indication that this latter culture is less likely to "throw off" variants, or it may be an indication that the variants produced are less noticeable than those produced by other cultures (7, 200).

The studies at Stanford University and the Carnegie Institution suggested that cultures producing more penicillin than the parent culture could be isolated from spore suspension that had received ultra-violet or x-ray treatment (58, 45, 197), and these two methods have been rather widely used (7, 200). This approach had been previously adopted by Hollaender (110, 111) in culture NRRL 832 with some success, but the results were not so striking as noted with the progeny of culture NRRL 1951, cultures X-1612 and Wisconsin Q_{176}; this earlier observation has often been overlooked. Further improvement was noted in a culture isolated when spores of Wisconsin Q_{176} were exposed to ultraviolet light for approximately one hour without stirring (88, 180). This culture, which produces more than two and one-half times as much penicillin as the parent, was also different from it in several physiological characteristics. Irradiation of it resulted in isolation of somewhat better cultures in line with observations previously cited.

Treatment of spore suspensions with a number of chemical agents thought to induce mutations in fungi has also been tried. The agents tested include camphor (135, 208, 209), colchicine (135, 210), nitrogen mustard (200, 210) and chloral hydrate (135). These studies have resulted in cultures producing more or less penicillin than the parent culture, but the improvement has not been so striking as when irradiation has been used.

Exposure of spore suspensions to neutrons (100, 170) and inclusion of radio-active phosphorus in the medium (117) have also been tested as means of isolating new high-producing cultures. Here again, while improved cultures were noted the improvement was less than that noted with ultra-violet irradiation, and the latter method appears to be the method of choice at present.

PIGMENT PRODUCTION BY PENICILLIN-PRODUCING CULTURES. Many of the cultures of the *P. notatum-chrysogenum* group which produce penicillin also produce a number of yellow pigments (43, 79), and, although pure penicillin crystals are colorless, most of the relatively impure preparations distributed prior to 1946 had a decided yellow tinge which caught the attention of the public and was popularly associated with penicillin. The yellow pigments may be made colorless by reduction with mercury, or they may be removed by chemical treatments (41*a*, 45, 54), but these steps are quite expensive in that a significant quantity of penicillin may be lost during the processing. However, as the manufacturers of penicillin preparations improved their processes the color of the final product became lighter with increased purity, although the crystals often have a slight yellow tinge. When it was realized (41, 255) that pure penicillin would be colorless, search for penicillin-producing cultures which did not produce these pigments was intensified. Several of the cultures isolated from soil samples studied at the University of Minnesota gave significant quantities of penicillin and at the same time did not produce the yellow pigments. However, they were relatively poor penicillin producers and were not widely used, since the manufacturers apparently were not in a position to reduce production in order to produce a purer product.

Recently high penicillin-producing variants have been isolated from the Wisconsin Q_{176} culture which do not produce these yellow pigments when grown on agar or liquid media (7, 25). Further isolations from these cultures by various techniques have resulted in a number of cultures which do not display these pigments and at the same time produce more penicillin than the parent culture (7, 139) when grown in shaken flasks on a corn-steep-lactose medium supplemented with β-phenylethylamine. These cultures also produce only penicillin-G on this medium and thus differ from the parent culture (Wis. Q_{176}) in another respect, for the latter culture produces only 80% to 90% penicillin-G on this medium (7, 123, 254). If these cultures become widely used in the industry it will be a rather major change from the pigment-producing multi-penicillin-producing cultures used within the past few years.

PENICILLIN PRODUCTION BY CULTURES NOT BELONGING TO THE *Penicillium notatum-chrysogenum* GROUP. While attention has been concentrated on penicillin production by the *P. notatum-chrysogenum* group and cultures of this group seem to be characterized by penicillin production (192, 195, 243), many other *Penicillium* cultures have been reported to produce penicillin. Penicillin-like substances have been isolated from cultures of *P. avellaneum, P. rubens, P. turbatum* (73), *P. lanosum, P. roseo-citreum, P. griseo-fulvum* and *P. spinulosum* (195). It is interesting to note that cultures of *P. turbatum* and *P. spinulosum* have been found to produce penicillin and the antibiotic substance spinulosin (195).

Penicillin or penicillin-like substances have also been reported as metabolic products of a number of Aspergilli, including *Aspergillus flavus* (151, 152, 195, 242), *A. parasiticus* (4, 47), *A. nidulans* (61, 62, 82, 195), *A. giganteus* (184), *A. niger* (82), *A. flavipes* (251, 82, 195) and *A. oryzae* (82, 252). It has also been noted in culture filtrates from *Trichophyton mentagrophytes* (177) and a thermophilic fungus (205). Identification of penicillin as a metabolic product in these studies has usually been on the basis of antibiotic spectra and heat stability, and the antibiotic has rarely been isolated in pure form. Production by these cultures apparently depends in part on the media used, as indicated in the studies with *A. parasiticus* (4, 47) where much higher antibiotic activity was noted when cornsteep water-containing media were used than when other media were employed.

While it is possible that further study of some of the above cultures will indicate that they may be more desirable than the strains of *P. chrysogenum* now in use, perhaps after selection procedures similar to those associated with the *P. chrysogenum* cultures, it is not too likely that the penicillin-producing industry will quickly transfer its operations to utilize these cultures. This reluctance to make such a change probably stems from the difficulty in revising recovery operations and other details associated with the production of penicillin which are only indirectly connected with the fermentation operations.

MEDIA USED FOR THE MYCOLOGICAL PRODUCTION OF PENICILLIN

HISTORICAL DEVELOPMENT. Successful production of large quantities of penicillin has depended in part on selection of cultures, as previously mentioned, and in part on the formulation of media suitable for the growth of these cultures. Fleming (70) grew his culture on a nutrient broth medium and noted that addition of glucose or sucrose to this medium apparently delayed production of the antibiotic principle. A modification of the Czapek-Dox medium (glucose and inorganic salts) was used by Clutterbuck et al. (43), and studies by the Oxford group (2) indicated that the sodium nitrate component of this medium could be replaced by peptone, the glucose by maltose or sucrose, and the proportions of the inorganic salts could be varied without marked effect on penicillin production. They also noted that addition of ten grams of yeast extract per liter to the medium resulted in a faster rate of production but apparently did not affect the quantities produced.

In other early reports (pre-1942) a number of other media were used, including veal broth (202), nutrient broth (20), tryptone-glucose-salts broth (69), amino acid mixture—brown sugar (69), a modified Czapek-Dox medium supplemented with peptone (150) and a modified Czapek-Dox medium in which brown sugar replaced glucose (150). In all of the studies (surface culture process) comparatively small quantities of penicillin were produced, usually only one to four units per ml. It was also generally observed that the penicillin-producing period was rather short, and if the pH of the fermentation was allowed to rise rapidly only small amounts of penicillin were found.

Perhaps the first outstanding improvement in the production of penicillin was associated with the observation made by Dr. A. J. Moyer in 1941 that addition of a small quantity of cornsteep liquor to the medium resulted in a manifold increase in penicillin production (44, 45, 159, 163). This material had been previously used as a component of many fermentation media, a few of which are noted in the review by Liggett and Koffler (144), and was available in large quantities. During the period extending from 1942 to the present it has apparently been widely used as a major constituent of the media for production of penicillin and other antibiotic substances by microorganisms, and although other media have been proposed on which the mold will grow well and produce penicillin, none of these has apparently replaced the cornsteep liquor medium (88*a*). Further studies by Dr. Moyer and associates indicated that penicillin production on a medium containing

TABLE V

GENERAL ANALYSIS OF CORNSTEEP LIQUOR

Constituent	grams/100 grams
Water	45–55
Total N (Kjeldahl)	2.7–4.5
Amino N (Van Slyke)	1.0–1.8
Volatile N	0.15–0.40
Free reducing sugar as glucose	0.1–11.0
Lactic acid	5–15
Volatile acids as acetic	0.1–0.3
SO_2	0.009–0.015
Ash	9–10
Some components of ash:	
Ca	0.25–0.75
Cu	0–0.0015
Fe	0.005–0.025
Mg	0.25–0.5
Mn	0.002–0.005
S	0.15
Zn	0.00025–0.0025
K	0.5–1

Abstracted from Liggett and Koffler (144) lactose as a carbohydrate source and cornsteep liquor as a nitrogen source was very high, and this medium has been used with some variations in most of the industrial production units (88*a*).

While this lactose-cornsteep liquor medium is relatively inexpensive and large quantities of the ingredients are available (44), a considerable amount of research has been expended in studying other media. Most of these media have contained a protein nitrogen source, a carbohydrate source, a mineral supplement and other ingredients, as will be mentioned in the following discussion. Several synthetic media in which the composition of all components is known have also been prepared, and, as will be indicated, penicillin production on a few of these has approached that on the cornsteep liquor–lactose medium, but none of these has been widely employed, usually for economic reasons.

NATURAL MEDIA. In discussing the various media which have been used for the culture of penicillin-producing fungi, it is desirable to divide them into two groups, the natural media and the synthetic media. At least one constituent of the former is a natural product whose exact composition is unknown, for example, yeast extract, cornsteep liquor, cottonseed oil meal. A synthetic medium is one whose composition is known, and, although it may contain ingredients of natural origin, these are highly purified and there is little chance that substances of unknown composition have been introduced. As in most media for the growth of fungi, the media used in producing penicillin have contained a nitrogen source, a carbohydrate source or energy source, and a number of minerals which are required for the growth of the fungus and indirectly perhaps for the production of penicillin. In the natural media protein nitrogen sources have been added rather than inorganic nitrogen, while in the synthetic media both organic and inorganic nitrogen-containing compounds have furnished the nitrogen required.

PROTEIN NITROGEN SOURCE. As has been mentioned above, the work of the Oxford group (2) indicated that addition of yeast extract at a level of 100 grams per liter to a basal medium containing per liter: sodium nitrate, three grams; monobasic potassium phosphate, one gram; potassium chloride, 0.5 gram; magnesium sulfate heptahydrate, 0.5 gram; ferrous sulfate heptahydrate, 0.01 gram; glucose, 40 grams; and tap or distilled water to one liter resulted in an increase in the rate of penicillin production but not in an increase in the quantity of this antibiotic. When the Fleming culture was grown on this medium (surface culture method) potencies of one to four units per ml. were obtained after a fermentation period of six to nine days. Addition of other supplements to this medium did not result in increased penicillin yields. Studies in other laboratories suggested that such materials as Amigen, a commercially available digest of purified casein and pork pancreas in which the proteins have hydrolyzed by enzmatic action, would serve as nitrogen source (233), but higher yields of penicillin were not obtained.

As has been mentioned, Dr. A. J. Moyer observed that cultures grown on the medium used by Abraham et al. (2) supplemented with two to five ml. of cornsteep liquor produced ten units per ml. of penicillin, and that increasing the concentration of cornsteep liquor in the medium to 100 to 125 grams per liter resulted in yields of 60 to 80 units (surface culture process) after other minor changes in the medium (159). Further experimentation indicated that the following medium perhaps contained the optimal concentration of ingredients for penicillin production of culture NRRL 1249.B21 (grams per liter): sodium nitrate, 3; magnesium sulfate heptahydrate, 0.5; monobasic potassium phosphate, 1; glucose monohydrate, 2.75; zinc sulfate heptahydrate, 0.044; manganese sulfate quadrahydrate, 0.004; lactose monohydrate, 44; cornsteep liquor, 100; and water to one liter. This medium resembles in part those used by earlier investigators and has been the basis for other studies in many laboratories. In some experiments yields approaching 200 units per ml. were obtained (159). The inclusion of cornsteep liquor is no doubt responsible for the increase in penicillin production, and perhaps a brief description of its manufacture, abstracted in part from Liggett and Koffler (144), is in order:

> "Since cornsteep liquor is a by-product of the wet-milling industry it would be insufficient to discuss its manufacture apart from the whole process in which corn, after having been shelled and air-cleaned, is soaked, and then fractionated into its principal components by a combination of flotation and wet-screening procedures".
>
> "The corn is first soaked, or steeped in open wooden tanks at 45–50° C for 40 to 48 hours. Five to seven gallons of water are required for every bushel of corn. The water used in steeping is process water that has been

used previously in other phases of the process, for example, the overflow from the gluten settling tank. During steeping the soluble materials are dissolved, the corn is softened, and its structure weakened and broken, which facilitates the grinding and further separation of its components. Just before the process water enters the tanks, SO_2 is added to prevent putrefaction and to assist in the extraction of the soluble compounds. The concentration of SO_2 is initially from 0.1 to 0.2%, but since most of the SO_2 is absorbed by the corn, it is lowered to 0.05% five hours after addition and to 0.01% within two hours. Moving in a general counter-current fashion, the most dilute water is placed on corn that has been steeped the longest and is transferred continuously in the direction of the corn most recently introduced. In this manner, the steep water having the highest concentration of solutes is used on corn just entering the system after which procedure the water is withdrawn and concentrated to a solid content of approximately 50%. This concentrated crude cornsteep liquor may then either be combined with gluten and fibrous materials and sold as animal feed, or used for microbiological purposes with or without further processing".

"The main disadvantage of cornsteep liquor in microbiology is its variable composition. This variability may depend somewhat upon the type and condition of corn, but even more upon a multitude of variables involved in the processing of starch. On the other hand, cornsteep liquor is an inexpensive alternative (4 to 6 cents per pound of liquor) to much more expensive materials such as yeast extract and peptone".

"Cornsteep liquor has a pH of 3.7 to 4.1, a specific gravity of 1.25, on proximate analysis a general composition as indicated in Table V. On the average 6.9% of the corn solids and 30% of the corn nitrogen are found in the steep liquor. Approximately 95% of the total nitrogen in cornsteep liquor is accounted for, after hydrolysis, by ammonia and the following amino acids: alanine, arginine, aspartic acid, cystine, glutamic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, threonine, tyrosine, and valine, with more than one-quarter of the nitrogen present as alanine (34). It also contains considerable amounts of the B-complex vitamins with the exception of thiamine (232). The ash of cornsteep liquor contains a wealth of mineral mineral elements, especially calcium, iron, magnesium, phosphorus, and potassium".

While the penicillin industry has used large quantities of cornsteep liquor during the past few years, the demand for this purpose has not exceeded more than 15% of that produced, with major production going to the live-stock feed industry. Under these conditions its has been rather difficult for the penicillin producers to arrange for supplies of this material which are of reasonable constant composition, and some variations in penicillin production have been attributed to poor cornsteep liquor (45). This was especially noticeable in 1945 when a considerable drop in the total production of penicillin in the United States occurred during the early fall months when the cornsteep liquor made with the poorer grade corn was being used in the preparation of media. Some effort has been made to produce a product which is standardized and perhaps better suited for inclusion in media used for the production of penicillin by alkali, fermentation and/or heat treatments (165, 169, 234). Some improvement in penicillin production of media prepared with poor cornsteep liquor is claimed by treatment of the medium with activated carbon (33).

The increase in penicillin yields noted after addition of cornsteep liquor to the culture medium was so marked that some effort was expended in investigating the specific role of the liquor in the metabolism of the mold and the production of penicillin. The liquor, as can be seen from Table V, supplies large quantities of nitrogenous materials, minerals (including the trace elements) and, as will be mentioned later, compounds which act as penicillin precursors. Addition of minerals and growth factors to media containing cornsteep liquor did not result in increased penicillin production in surface culture (159). Fractionation of the liquor by a number of procedures did not indicate which factors were responsible for the promotion of penicillin production (159), although some stimulation was obtained when certain amino acids present in cornsteep liquor were added to synthetic media (31*a*, 99*a*, 252). Later investigations indicated that the liquor contains appreciable quantities of phenethyl and phenacetyl derivatives which probably act as precursors for the production of penicillin, but this was not recognized until after considerable work had been done on the chemistry of the penicillins, and perhaps should be considered a by-product of these latter studies. As has been mentioned earlier, increasing the concentration of cornsteep liquor in media used for surface culture resulted in increase in penicillin production, and, as indicated in Table VI, this same effect was observed when submerged culture methods were used. Addition of a buffering agent is apparently desirable in the submerged culture method (84, 160, 161), while it may not be necessary in the surface culture process (159), and, as indicated in Table VI, calcium carbonate has been found suitable for this purpose.

A large number of naturally occurring products have been tested for penicillin-promoting activity similar to that noted when cornsteep liquor was added to the medium. The results obtained in various laboratories have varied somewhat with the cultures and processes used, but in general indicate that addition of such materials as hydrolyzed casein, hydrolyzed corn, wheat or soybeans to a basal medium containing inorganic salts and carbohydrate did not stimulate penicillin production to the extent noted when cornsteep liquor was added (79, 84, 159, 160, 162, 163, 164, 93). This once was interpreted as an indication that some factor present in cornsteep liquor is essential for the production of penicillin (22), but later studies with synthetic media and with media prepared with seed meals indicate that cornsteep liquor is not exceptional in its penicillin-promoting ability (85, 87, 79, 179, 93). Indeed, as indicated in Table VII, penicillin production may at times be higher on media prepared with certain seed meals than with media prepared with cornsteep liquor. Less promising results have been obtained with chestnut extract (173) and pea extracts (47, 48, 49, 50, 51), and, while peptone has been found suitable in one instance (236) it has not been useful in others (159, 179) when compared with cornsteep liquor. Some of these

TABLE VI

EFFECT OF NUTRIENT LEVEL ON PENICILLIN PRODUCTION

Composition of medium				Maximum penicillin yield	Age at maximum yield
Cornsteep liquor (solids)	Lactose	$CaCO_3$	Salts*		
g/100 ml	g/100 ml	g/100 ml		units/ml	hours
2	2	0.2	+	237	54
4	3	1	–	480	64
6	3	1	–	636	67

*Salt mixture (grams/l): KH_2PO_4, 0.25 g; $MgSO_4 \cdot 7H_2O$, 0.12 g; $NaNO_3$, 1.5 g; at low cornsteep liquor concentrations the use of an auxiliary nitrogen source is desirable.

Fermentations conducted in 80 gallon tanks containing 220 l. of medium with aeration at rate of 200 liters per minute, and agitation by 12 in. propellors revolving at 270 r.p.m. and temperature control at 23°C.

Penicillium chrysogenum X-1612.

Abstracted from Johnson et al.(121).

materials, notably the seed meals, are available in large quantities and are perhaps of less variable composition than the cornsteep liquor and may find use as ingredients of media for the production

TABLE VII

PRODUCTION OF PENICILLIN ON NATURAL MEDIA

Substance substituted for cornsteep liquor*		Maximum penicillin without precursor	Production with precursor
	g/l	U/ml	U/ml
Cornsteep liquor (solids)	20	650	1,300
	30	580	1,050
	40	400	800
Cottonseed oil meal	30	800	1,200
	40	1,000	1,160
Linseed oil meal	20	700	1,035
	40	635	1,100
	60	...	570
Coconut oil meal	60	450	1,150
	90	500	810
	120	300	680
Sardine meal	10	150	610
	20	400	900
	30	575	1,130
Rape seed oil meal	30	285	515
	60	730	705
	90	335	615
Peanut oil meal	20	600	630
	30	950	900
	40	1,050	1,350
Mustard flour	30	650	980
	40	590	1,060
Soybean meal (expeller).	20	305	1,030
	40	170	930
Castor bean meal	20	225	940
	40	300	580
Fish meal (mixed)	20	830	430
	40	540	790
Menhaden meal	20	780	390
	40	460	430

*Other ingredients included 30 g. lactose and 1 g. phenylacetylethanolamine as penicillin G precursor (where indicated), 3.2 ml. soybean oil, and 10 g. calcium carbonate per liter.

Adapted from Perlman (179).

of penicillin in certain sections of the world where cornsteep liquor is not available.

Carbohydrate Constituents and Energy Sources. When the Czapek-Dox medium (2) or slight modifications of it (43, 59, 79, 150) were used in the surface culture process there was no marked difference in response to various carbon sources, as indicated by penicillin production, except that lactose was inferior because it supported only a trace of fungus growth (159). The slight increase resulting by substitution of brown sugar (86, 150) for the glucose could be attributed to the presence of metallic impurities in the brown sugar. However, once the effectiveness of cornsteep liquor was recognized and this material was added to the basic medium, a marked effect of various carbon sources on penicillin yield was observed (159). There was sufficient assimilable carbohydrate, probably mainly glucose and dextrins, to support good fungus growth and moderate penicillin production, even in the absence of added carbon sources. Lactose, corn starch, and corn dextrin were equally good for penicillin production (159, 176, 168) with glucose, glycerol, sorbitol and brown sugar (sucrose) being significantly inferior. Crude starch preparations, including ground corn, ground wheat and granular wheat flour, could be used in this surface culture process (159). In one report addition of corn oil to a modified Czapek-Dox medium containing brown sugar as a glucose replacement resulted in a significant increase in penicillin production, while addition of lactose to this medium did not (112).

In certain laboratories comparable studies on carbohydrate sources in submerged cultures suggested that higher penicillin potencies were obtainable when a cornsteep–brown sugar medium was used instead of a cornsteep-lactose or a cornsteep-glucose medium (84, 86). Sucrose and glycerol could be substituted for brown sugar without appreciable change in penicillin production, but replacement of brown sugar with cane molasses and other crude sucrose sources was not always satisfactory. In other laboratories higher yields were obtained with a cornsteep-lactose medium (44, 160, 164), and apparently this medium served in some manufacturing plants, while the cornsteep liquor–brown sugar medium was used in others. This apparent discrepancy between results in different laboratories may be partially explained by the studies on the effect of aeration on penicillin production when the fungus was grown on these different media (135, 182), and is probable that there were considerable differences in aeration efficiency in the various penicillin-manufacturing plants. Among the carbohydrates that may be substituted for lactose are sorbitol, mannitol and *a*-methyl glucoside, while starch, inulin, fructose, glucose, galactose, sorbose and xylose do not appear to serve as satisfactory substitutes (178, 179). Crude lactose sources, including cheese whey, may be used as replacements for the purified product (179) without appreciable effect on penicillin yield, while casein whey apparently cannot be employed for this purpose (179). Lactose is metabolized slowly under these aeration conditions (135, 178) and requires less oxygen for utilization, while simpler sugars are metabolized rapidly and require higher aeration rates. Mixtures of lactose and glucose or lactose and sucrose apparently may be substituted for lactose without appreciable effect on penicillin production (178). The fungus produces amylases and invertase which hydrolyze the polysaccharide and sucrose quickly (85, 178) but apparently does not produce lactase or an enzyme splitting *a*-methyl glucoside (178).

TABLE VIII

EFFECT OF ADDITION OF PHENYLACETYL DERIVATIVES ON THE PRODUCTION OF PENICILLIN*

Phenylacetyl derivatives added**	Units of penicillin per ml	Penicillin G content of penicillins produced
None	242	0
Phenylacetic acid	490	82
Phenylacetamide	504	71
Phenylacetyl-*dl*-alanine	483	57
Phenylacetyl glycine	461	56
β-phenylethylamine	428	51
dl-phenylalanine	220	24

*Abstracted from Singh and Johnson (218).

**Quantity of derivative equivalent to 0.14% phenylacetic acid was added to cultures of *Penicillium chrysogenum* Wisconsin Q_{176} (growing on synthetic medium) 24 hours after inoculation with a vegetative inoculum.

INORGANIC CONSTITUENTS. The ash content of cornsteep liquor supplies a considerable quantity of minerals to the medium, and supplementation of media containing this material with trace elements and other salts has not resulted in considerable change in penicillin production (159). As has been mentioned before, Dr. Moyer added traces of zinc sulfate and manganese sulfate to his media, but no definite need could be established for these minerals when the basal medium contained cornsteep liquor (144). Addition of iron salts to the cornsteep liquor medium, either as an ingredient of the medium or by erosion from iron equipment, resulted in decrease in penicillin yields when more than 30 p. p. m. were present (226). Addition of sodium nitrate, magnesium sulfate and potassium phosphate (85, 159, 160) did result in increase in penicillin production, and several studies have been made to determine the optimum concentrations of these ingredients for use in the surface culture (186, 187) and the submerged culture process (188). However, as penicillin production was not greatly decreased when these materials were eliminated from cornsteep water–lactose or seed meal–lactose (22, 179) media, it is probable that they are not of major importance, although it may be desirable to add them to supplement those quantities of nitrogen, sulfur and phosphorus already in the medium from the cornsteep liquor. Addition of borax to a cornsteep water–lactose medium appeared to stimulate penicillin production by several cultures of *P. chrysogenum* but had little effect on others (127, 136, 137*a*).

Addition of calcium carbonate to cornsteep liquor–lactose media in the submerged culture process has already been mentioned. Substitution of other alkali metal carbonates in these media for calcium carbonate did not result in as high penicillin yields as with calcium carbonate (178), and use of sodium or potassium hydroxide to control the pH of the media resulted in decreased penicillin production (121, 226). While this may suggest a calcium requirement for the mold, the high yields obtained when synthetic media, which contain only trace amounts of calcium salts, are used, do not substantiate this hypothesis.

PENICILLIN PRECURSORS. As the chemistry of the penicillins was examined during the early days of production development, it soon became evident that more than one compound exists which has the pharmacological properties associated with the name "penicillin". American workers isolated a pure crystalline penicillin termed "penicillin-G" (41, 255), which was characterized by having phenylacetic acid as a degradation product, whereas the penicillin studied by the English workers, termed "penicillin-F", contained the Δ-2-pentenyl radical, as indicated in Table I, and did not yield phenylacetic acid as a degradation product. Moyer and Coghill (46, 161), in studying the production of penicillin in surface and submerged culture, added a number of degradation products of penicillin to the culture medium. When phenylacetic acid was added an increase in the total quantity of penicillins produced was noted, and the proportion of penicillin-G also seemed to increase, as measured by bioassay and isolation procedures. In these studies the quantity of phenylacetic acid converted to penicillin-G was less than 2% of that added to the fermentation, but this observation was of great practical importance, for it permitted direction of the fermentation to the production of the desired penicillin-G, and it is probable that all media now used commercially contain added penicillin-G precursor.

Studies in other laboratories (9, 41) utilizing phenylacetic acid marked with deuterium established that some of the phenylacetic acid added to fermentations was converted eventually to penicillin-G. Later experiments (55) have indicated that if phenylacetamide labeled with C^{13} at the amide group is added to a growing culture, the penicillin-G isolated from this culture will contain the isotopic tracer in the expected position. These labeled penicillins have been used in analysis of penicillins for penicillin-G by the isotopic dilution method.

A number of derivatives of phenylacetic acid and related compounds have served in these studies, including phenylethylamine, phenylacetamide, phenaceturic acid, phenylacetylethanol amine and derivatives of phenylacetamide (41, 85, 87, 88, 95, 103, 142, 143, 155, 186, 190, 218, 237). The efficiency of the conversion of some of these compounds to penicillin-G as calculated from the data presented in Table VIII, is less than 5%. This varies somewhat from one compound to another and also probably depends in part on the strain of *P. chrysogenum* and the cultural conditions (260). Addition of phenylacetylethanol amine to the culture medium has also been shown to increase the production of an unidentified antibiotic, presumably penicillin-G, by cultures of the *Aspergillus nidulans* group (62).

This observation that addition of phenylacetic acid to growing cultures resulted in formation of penicillin-G prompted Behrens and associates (9–15) to add other compounds to the medium, including substituted phenacetyl derivatives, containing halogens and other groups, and other organic radicals. They were successful in isolating a number of penicillins in crystalline form which contained the added material in the R-position (see Table I) and which apparently did not occur naturally when the fungus was grown on the usual media. These are often termed "artificial penicillins" to distinguish them from those called "natural penicillins," produced when the fungus is grown on synthetic or cornsteep-liquor-containing media. Obviously the distinction is rather arbitrary. Recent experiments (25) have indicated that addition of some of the *n*-fatty acids or glycerides of these fatty acids to synthetic or cornsteep-water-containing media resulted in production of the corresponding penicillin containing an aliphatic grouping in the R-position.

The difference in the types of penicillins produced in the United States and England was eventually explained by the observation that cornsteep water which was widely used in the former country contains appreciable quantities of phenethylamine and phenylalanine (41, 88, 154). These compounds apparently act as penicillin-G precursors and account for the penicillin produced when cultures are grown on cornsteep-water-containing media. It is probable that the phenylalanine content of the protein constituents of the seed meal media (85, 87, 179) acts in the same manner. Present indications are that practically no penicillin-G is produced when cultures of *P. chrysogenum* are grown on synthetic media which do not contain added phenylacetyl derivatives (123), although the mold mycelium contains approximately 2% phenylalanine (182). If this is found to be generally true, penicillin-G should be really classified as an artificial penicillin and probably would have never been found if Dr. Moyer had not added cornsteep water to his media.

Cornsteep water was also found to have some tyramine, presumably derived by bacterial decarboxylation of tyrosine, which was assumed to serve as a precursor for the production of penicillin-X (88, 154). Addition of tyrosine or tyramine to growing cultures resulted in the production of penicillin-X (5, 35, 95, 142, 143, 155). As has been mentioned previously, certain cultures appear to be predisposed to the production of one penicillin over another, and perhaps the most notable is the group producing large quantities of chloroform-insoluble penicillin-X (194, 196–198).

While a number of compounds have been studied as possible precursors for the other portions of the penicillin molecule, no reports have demonstrated that any compounds have such activity when added to growing cultures (9, 229, 230), although addition of a number of amino acids has resulted in some increase in penicillin production which may be through this mechanism (229, 231).

SYNTHETIC MEDIA. Concurrent with the studies on the production of penicillin by fungi grown on natural media, as mentioned above, investigations were in progress studying penicillin production by these fungi when grown on synthetic media. A large number of media formulations were tested with varying results. The composition of several of the more successful from the point of view of penicillin production are listed in Table IX with some indication of the antibiotic yields obtained. The highest yields of penicillin were achieved on media which resembled in part the cornsteep liquor–lactose media in that they contained those ingredients or similar materials found in the cornsteep water. Although the yields on these synthetic media approach those obtained on the cornsteep-liquor media, no reports are available which suggest that they have been used in larger than pilot-plant units.

While most cultures of *Penicillium chrysogenum* will grow rather well on a number of synthetic media, penicillin yields are not always associated with good growth, as measured by quantity of mycelium formed. Jarvis and Johnson (114–116) have evaluated the role that certain constituents of a synthetic medium play in the production of penicillin. Their observations led them to conclude that the rate of formation of penicillin appeared to reach a maximum under conditions which supported only a very slow growth rate. As indicated in Table IX, they obtained yields of over 300 units per ml. on a medium which contained lactose, glucose, ammonia, acetate, lactate and inorganic salts, and after supplementation with a penicillin-G precursor, yields increased to

TABLE IX

PENICILLIN PRODUCTION ON SYNTHETIC MEDIA

A. Surface Culture Process			
Composition of medium:	lactose, 30 g glucose, 10 g citric acid, 15 g acetic acid, 2.5 g phenylacetic acid, 0.5 g $MgCl_2 \cdot 6H_2O$, 0.5 g $FeCl_3$, 0.05 g $Cu(NO_3)_2 \cdot 3H_2O$, 0.02 g $ZnCl_2$, 0.02 g $MnCl_2 \cdot 4H_2O$, 0.02 g $Co(NO_3)_2 \cdot 6H_2O$, 0.02 g Na_2SO_4, 1 g NH_4NO_3, 5 g NaF, 0.01 g Distilled water to 1 liter (pH adjusted to 5.8—with KOH)	lactose, 30 g glucose, 10 g starch, 15 g acetic acid, 2.5 g citric acid, 10 g phenylacetic acid, 0.5 g $(NH_4)_2SO_4$, 5 g Ethylamine, 3 g $MgSO_4 \cdot 7H_2O$, 0.5 g KH_2PO_4, 1 g $CuSO_4 \cdot 5H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.02 g $ZnSO_4 \cdot 7H_2O$, 0.01 g $MnSO_4 \cdot 4H_2O$, 0.01 g $Co(NO_3)_2 \cdot 6H_2O$, 0.02 g NaF, 0.01 g Distilled water to 1 liter (pH adjusted to 5.6–5.9 with KOH)	lactose, 15 g glucose, 15 g $NaNO_3$, 6 g KCl, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g Succinic acid, 10 g Glutamic acid, 4 g Arginine, 0.3 g Histidine, 0.3 g KH_2PO_4, 1.5 g $ZnSO_4 \cdot 7H_2O$, 0.001 g $FeSO_4 \cdot 6H_2O$, 0.010 g $MnSO_4 \cdot 5H_2O$, 0.01 g $CuSO_4 \cdot 5H_2O$, 0.005 g Distilled water to 1 liter (pH adjusted to 5.7–5.8)
Inoculum:	Spore suspension	Spore suspension	Spore suspension
Volume of fermentation:	100 ml	100 ml	100 ml
Culture used:	NRRL 1249.B21	NRRL 1249.B21	NRRL 1249.B21
Maximum yield:	70 Units/ml	119 Units/ml	205 Units/ml
Reference:	107	31a	252
B. Submerged Culture Process			
Composition of medium:	lactose, (monohydrate), 22.5 g glucose, 7.5 g ammonium acetate, 3 g ammonium lactate, 5 g KH_2PO_4, 3 g $MgSO_4 \cdot 7H_2O$, 0.25 g $FeSO_4 \cdot 7H_2O$, 0.1 g $CuSO_4 \cdot 5H_2O$, 0.005 g $ZnSO_4 \cdot 7H_2O$, 0.02 g Na_2SO_4, 0.5 g $MnSO_4 \cdot 4H_2O$, 0.02 g $CaCl_2 \cdot 2H_2O$, 0.05 g distilled water to 1 liter	lactose, 30 g glucose, 10 g ammonium acetate, 3.5 g ammonium lactate, 5.5 g KH_2PO_4, 3 g $MgSO_4 \cdot 7H_2O$, 0.25 g $FeSO_4 \cdot 7H_2O$, 0.1 g $CuSO_4 \cdot 5H_2O$, 0.005 g $ZnSO_4 \cdot 7H_2O$, 0.02 g Na_2SO_4, 0.5 g $MnSO_4 \cdot 4H_2O$, 0.02 g $CaCl_2 \cdot 2H_2O$, 0.05 g phenylacetic acid (in 9 additions during fermentation), 14.5 g distilled water to 1 liter	lactose, 15 g glucose, 5 g acetic acid, 4 g NH_4NO_3, 5 g KNO_3, 3.5 g KH_2PO_4, 2 g $MgSO_4 \cdot 7H_2O$, 0.5 g $FeSO_4 \cdot 7H_2O$, 0.2 g $ZnSO_4 \cdot 7H_2O$, 0.04 g $CuSO_4 \cdot 5H_2O$, 0.005 g phenylacetamide, 0.25 g distilled water to 1 liter pH adjusted to 6 with KOH

TABLE IX (continued)

Inoculum:	3% of vegetative growth grown on glucose-salts medium	1.5% of vegetative growth grown on glucose-salts medium	10^5 germinated spores grown on cornsteep water medium
Size of fermentation:	80 ml in 500 ml Erlenmeyer flask	80 ml in 500 ml Erlenmeyer flask	50 gallons in glass-lined tank
Culture:	*P. chrysogenum* Wis. Q_{176}	*P. chrysogenum* Wis. Q_{176}	*P. chrysogenum* Wis. Q_{176R}
Maximum penicillin yield:	360 U/ml	915 U/ml	245 U/ml
Reference:	116	218	97

over 500 units per ml. (116, 218). Analyses of the penicillin types present in media in which these precursors were not added indicated that little penicillin-G was produced in the absence of a phenylacetyl derivative (103, 104, 116, 123, 218). This suggests that the major portion of the penicillins produced on other synthetic media (2, 41*a*, 75*a*, 79, 99*a*, 126*a*, 137*a*, 137, 189, 190, 231, 252) was probably of the aliphatic type. Such a conclusion is difficult to substantiate, as the analytical methods used during the period 1943–1946 were not very specific with regard to determination of certain types of penicillins.

A rather large number of observations were made on the effects of certain inorganic elements on the production of penicillin by cultures grown in both surface and submerged culture processes (41*a*, 85, 103, 116, 126*a*, 137, 137*a*, 127, 190, 178). Addition of the ash obtained from cornsteep water (126*a*, 127) or from cottonseed oil meal (84) to synthetic media resulted in increased penicillin production. The activity of the former preparation was found to be associated with its iron and phosphate content (137*a*). A requirement for other elements, e. g., chromium, has been noted in one laboratory (189, 190) but has not been confirmed elsewhere (116, 137*a*). A number of sulfur compounds have been tested (35, 107, 142, 149) and, while poor growth was noted on a sulfur-free medium, inorganic sulfate was as effective as any compound in stimulation of growth and penicillin production.

Quantitative studies have been made (116*a*) on the sulfur, potassium, magnesium, phosphorus and iron requirements of *Penicillium* *chrysogenum* Q_{176} for the production of penicillin when grown on a synthetic medium. The iron, phosphorus and sulfur requirements for normal penicillin production were, respectively, 20 times, two times, and about one and one-half times those for normal growth. For potassium and magnesium, normal penicillin production was obtained at the same metal level as that required for normal growth.

Supplementation of synthetic basal media with some of the amino acids found in cornsteep water has resulted in some increase in penicillin production under certain conditions (31*a*, 51, 99, 229, 231, 252, 256, 257). Study of these reports suggests that perhaps the basal media used in these experiments were deficient in some essential nutrient, although it is possible that some of these substances are more easily assimilated as carbon and nitrogen sources than as ammonium lactate or ammonium acetate.

STUDIES ON THE BIOSYNTHESIS OF THE PENICILLINS

Studies on the metabolism of the penicillin-producing fungi were started shortly after the development of media which supported growth and penicillin production. These studies included observations on the chemical changes brought about on the medium constituents by the growth of the mold, study of some of the enzyme systems of the penicillin-producing fungi, investigation of the effects of various physical conditions on the metabolism of the fungus and penicillin production, testing of certain compounds as possible precursors or intermediates in the biosynthesis of the penicillins, and preliminary study of biosynthesis of possible intermediates in the biosynthesis of the penicillins.

As will be indicated below, a considerable amount of time has been spent studying the chemical changes occurring during the fermentation of various types of media, trying to correlate these changes with penicillin production. Unfortunately the information collected has not indicated at just what stage in the metabolism of the mold penicillin is formed, and actually little more is known of the mechanism of penicillin formation than was known ten years ago. However, these studies have served to explain in part why penicillin production on certain media is high or low under certain physical conditions, and this information has been of some value in the manufacture of penicillin. After surveying the reports which have appeared concerning this topic, it is apparent that much remains to be done before satisfactory explanations are available to explain why some cultures are good penicillin producers and others are poor ones.

CHEMICAL CHANGES OCCURRING DURING FERMENTATION OF VARIOUS MEDIA BY PENICILLIN-PRODUCING FUNGI. The many studies on the chemical changes occurring during the fermentation of various media by penicillin-producing fungi when grown in surface culture (31*a*, 79, 107, 108) and submerged culture (84, 92, 88*a*, 97, 114–116, 120, 135, 137, 138, 160, 179, 218, 226, 227) have indicated in part the conditions which are most favorable for penicillin production without apparently indicating what parts of the metabolic systems of the mold are actually involved in the production of penicillin. The role of certain constituents of synthetic (114–116, 127, 128, 136, 137*a*) and natural media (22, 179, 186, 187, 188) have been evaluated but have not shed much light on this problem. Usually the submerged culture fermentations may

TABLE X

CHANGES CHARACTERIZING THE THREE PHASES OF PENICILLIN FORMATION (LACTOSE-CORNSTEEP WATER MEDIUM)

	Phase 1	Phase 2	Phase 3
Penicillin	Slight production	Maximum rate of production	Concentration falls
pH	Sharp rise	Plateau or slight drop	Rise
Mycelium	Rapid growth, high N content	Slow growth, N content lower	Decrease in weight and N content
Lactose	Used slowly	Used more rapidly	Small remaining amount exhausted
Lactic acid	Exhausted rapidly		
Ammonia	Released into medium	Utilized	Released into medium
Nitrate	Used at maximum rate, although slowly	Slow use	Slow use, not exhausted
Nonammonia Kjeldahl N	Used extensively	Concentration rather stable	Concentration increases
Inorganic phosphorus	Used at maximum rate, although slowly	Slow use	No use or liberation
Q_{O_2} (N)	Maximum	Decreases	Minimum

Abstracted from Koffler et al. (135).

be divided into three phases, as indicated in Table X, on the basis of the metabolic changes noted, and combining these observations with measurements of the penicillin yields at various stages has permitted speculation on the optimum conditions for penicillin production (115, 120, 227). Sometimes the conditions postulated have been reproduced with high yields of penicillin, and unfortunately for these theories, at other times when these conditions have been met, penicillin yields have been low.

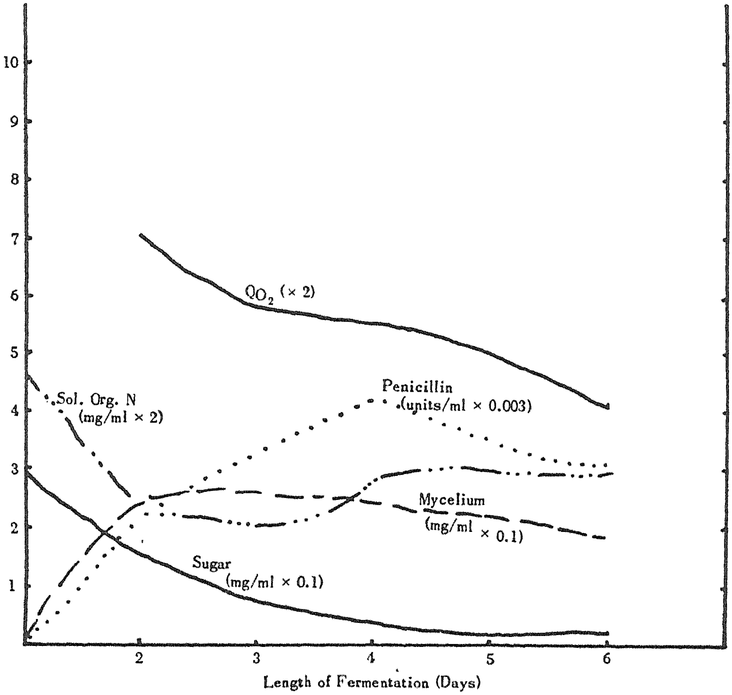


FIG. 3A. Chemical changes occurring during fermentation of cornsteep water medium by *Penicillium chrysogenum* Wisconsin Q_{176} (Shaken Flask). Medium composition (grams per liter): cornsteep water solids, 20; lactose, 30; $CaCO_3$, 10; N(2-hydroxyethyl)phenylacetamide, 1; soybean oil, 2.8; water to 1 liter. (Abstracted from Perlman (180)).

The changes occurring during fermentation of a cornsteep water–lactose or a cornsteep water–sucrose medium by non-penicillin-producing cultures were found to closely resemble those occurring during fermentation of these media by penicillin-producing cultures (135), suggesting that the changes noted were not important as far as penicillin production was concerned. Also, as indicated in Figs. 3A and 3B, the changes occurring when relatively poor penicillin-producing cultures are used are nearly the same as when good penicillin-producing cultures are studied, and it has apparently not been possible to select new cultures on any other basis than penicillin production. This situation is further complicated by the observations that certain cultures produce large amounts of one type of penicillin from a given medium, while others produce large amounts of another type from the same medium (7, 25–27, 194).

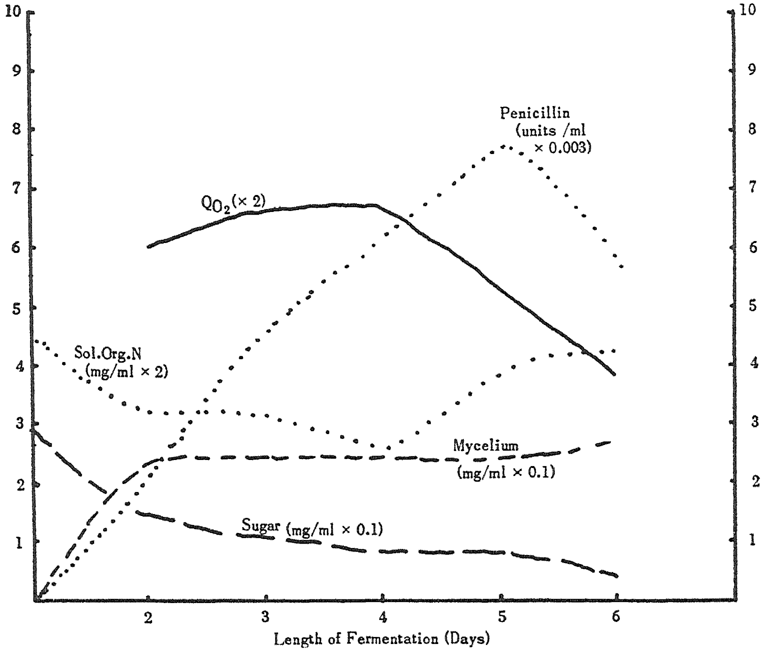


FIG. 3B. Chemical changes occurring during fermentation of cornsteep water medium by variant of *Penicillium chrysogenum* Wisconsin Q_{176} (Shaken Flask).

Medium composition (grams per liter) : cornsteep water solids, 20; lactose, 30; $CaCO_3$, 10; N(2-hydroxyethyl)phenylacetamide, 1; soybean oil, 3.2 ml.; water to 1 liter. (Abstracted from Perlman (180)).

In general the sequence of chemical changes noted in small fermentations conducted in shaken flasks (135, 179) has been observed in studies in large units (26–28, 120, 226). Some of the data collected in stirred jar fermenters are presented in Figs. 4 and 5. Alterations in the temperature of the fermentations (178, 226, 227), the aeration rates (26, 28, 97, 23), the methods of agitation and aeration (26, 27, 97, 226), and the medium composition (26, 28, 115, 116, 97) have some effect on the duration of some of these phases but have not been found to affect the sequence of these changes markedly (97, 115, 116, 135, 178). Such alterations as pH control by addition of acid or alkali (28, 226, 227) and continuous addition of ingredients (28, 160, 163) have at times

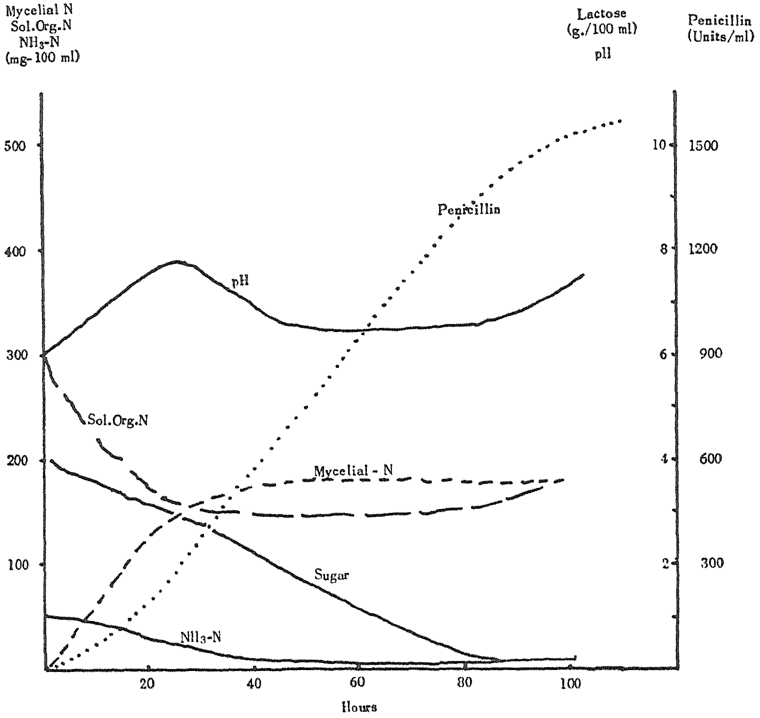


FIG. 4. Chemical changes occurring in stirred jar fermentations producing highest yields of penicillin from *Penicillium chrysogenum* Wisconsin Q_{176}. Medium composition (grams per liter): cornsteep water solids, 40; lactose, 2.5; glucose, 10; $CaCO_3$, 10; Na_2SO_4, 1; ammonium acetate, 1.5; water to 1 liter. (Abstracted from Peterson (82)).

prolonged the penicillin production phase slightly, but the results obtained in these experiments have not been so useful as had been expected. Study in flask and stirred jar fermentation units of the oxygen demand when various carbohydrates have been present in the media as energy sources has indicated (120, 135, 179, 256, 257) that higher aeration rates are required to obtain high penicillin yields when the media contain glucose, fructose, sucrose, starch and dextrin, singly or in combination, than when lactose, mannitol or α-methyl glucoside, which are more slowly oxidized by the fungus, are used (135, 178, 179, 257).

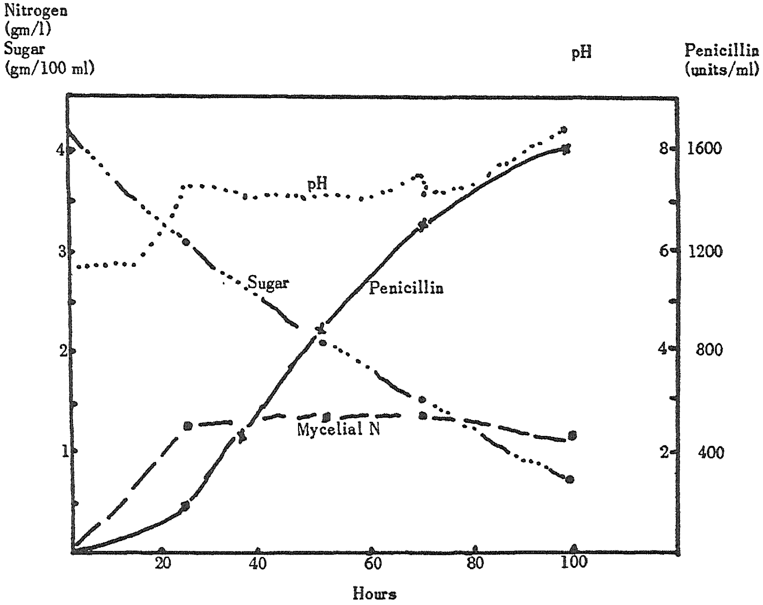


FIG. 5. Chemical changes occurring in stirred jar fermentation on *Penicillium chrysogenum* Wisconsin Q_{176} with relatively stable pH control.
Medium composition (grams per liter): cornsteep water solids, 40; lactose, 25; glucose, 9.1; Na_2SO_4, 1; $CaCO_3$, 5; phenylacetic acid, 3.5, added in 12 portions at 12-hour intervals; water to 1 liter. (Abstracted from Brown and Peterson (28)).

Most of the above studies were made on cultures grown on media containing cornsteep water as a nitrogen and partial carbon source. When linseed oil meal or cottonseed oil meal were substituted for the cornsteep water, approximately the same changes were noted (179) as had been found with the cornsteep-water-containing media. Formulation of several synthetic media, based in part on the composition of cornsteep water, has already been mentioned, and the chemical changes occurring during fermentation of these media resemble those noted in the cornsteep-water media, as indicated in Fig. 6. Use of synthetic media in these studies on the biosynthesis of penicillins seems advisable, as it probably permits easy reproduction of fermentations, an accomplishment that may be rather difficult when different natural media are used.

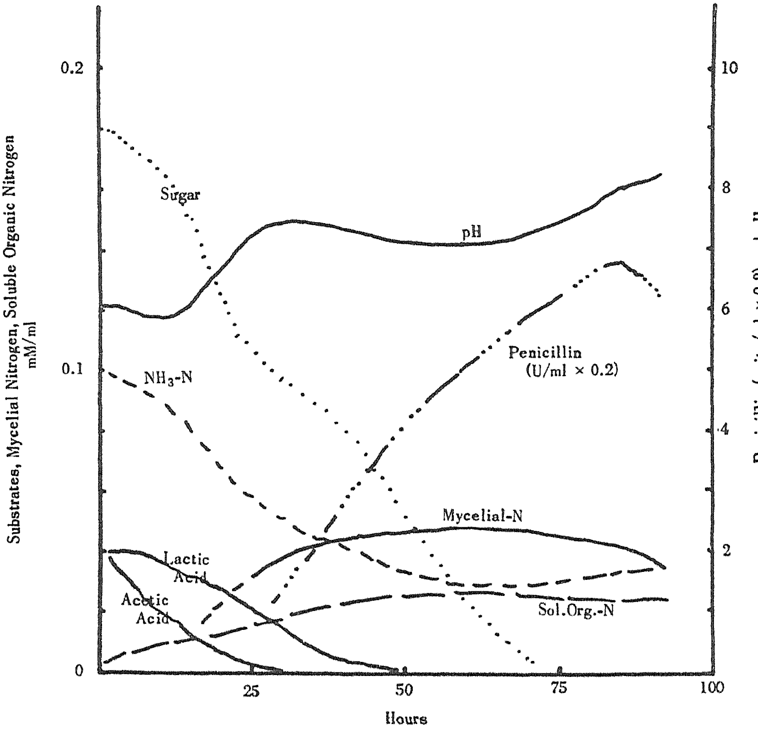


FIG. 6. Chemical changes occurring during fermentation of synthetic medium by *Penicillium chrysogenum* Wisconsin Q_{176} (Shaken Flask).

Medium composition (grams per liter): lactose, 22.5; glucose, 7.5; ammonium acetate, 3; ammonium lactate, 5; KH_2PO_4, 3; $MgSO_4 \cdot 7\ H_2O$, 0.25; $FeSO_4 \cdot 7\ H_2O$, 1; $CuSO_2 \cdot 5\ H_2O$, 0.005; $ZnSO_4 \cdot 7\ H_2O$, 0.02; Na_2SO_4, 0.5; $MnSO_4 \cdot H_2O$, 0.02; $CaCl_2 \cdot 2\ H_2O$, 0.05; distilled water to 1 liter. (Abstracted from Jarvis and Johnson (116)).

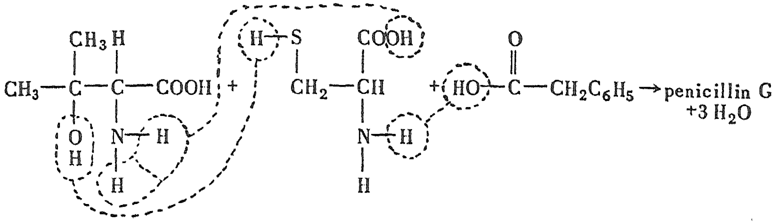
Most penicillin-producing cultures of *Penicillium chrysogenum* produce substantial quantities of proteolytic enzymes, amylases, phosphatases, amino acid oxidases, invertase and lipase during their growth on cornsteep-water media (84, 120, 128, 135, 139, 159, 179). While washed mycelium suspensions will produce some penicillin when incubated with a buffer–yeast extract solution (90), this approach to the mechanism of penicillin production has not received much attention. Apparently only preliminary studies have been made on the constitutive and extra-cellular enzymes of penicillin-producing molds, and no correlation between the activity of these enzymes and penicillin production has been postulated.

SEARCH FOR SUBSTANCES ACTING AS INTERMEDIATES IN BIOSYNTHESIS OF THE PENICILLIN. Mention has been made of the observations of Moyer et al. (46, 161) and Behrens et al. (9, 10, 11) that addition of phenylacetyl derivatives and other compounds resulted in increase in production of penicillins when added to growing cultures. These compounds have influenced the biosynthesis of penicillins from the R-group (see Table I), and, while successful and of considerable practical importance, the conversion of these compounds to penicillins has rarely exceeded 15% of that added and usually is of the order of 2% to 5%. It seems probable that the remainder of the preparations are oxidized or otherwise utilized by the mold as in animal tissues (24), and perhaps these studies may be interpreted to indicate that the fungus synthesizes excess quantities of the thiazolidine fragment.

Studies on the metabolism of sulfur compounds by penicillin-producing fungi have also been mentioned and have not indicated whether oxidized or reduced sulfur compound are more desirable for penicillin synthesis (5, 107, 108, 142). It is interesting to speculate that the variant of *Penicillium chrysogenum*, Wis. Q_{176}, which produces 3050 units of penicillin-G per ml., requires about .2 mg. per ml. of S to form enough thiazolidine portion of the molecule. This is nearly all the sulfur supplied by the 20 mg. of cornsteep water solids in the medium on a per ml. basis (144, 180).

Addition of penicilloic acid or other enzymatic (41, 161) or chemical breakdown products of penicillin to growing cultures has not resulted in increases in penicillin production, perhaps an indication that penicillinase action is not reversible by penicillin-producing organisms. While penicillinase activity has been noted in mycelial preparations of *P. notatum* NRRL 1249.B21, the organism used in the surface culture process, no activity has been noted in cultures grown under submerged conditions, although penicillin potencies in the broth drop rather sharply after the pH of the fermentation passes 8.3 (91, 84, 135). The effectiveness of certain metallic ions in inhibiting the production of penicillin by certain cultures (127, 137*a*) may be through activation of penicillinase as well as by the chemical catalysis of decomposition (37, 41).

Among the schemes suggested for the biosynthesis of penicillins is that of Hockenhull et al. (109) in which β-hydroxyvaline is thought to combine with cysteine and the penicillin precursor:



However, no evidence has been offered that these intermediates were synthesized by penicillin-producing cultures, although dimethylpyruvate was isolated from growing cultures and may have been an intermediate in the formation of the β-hydroxyvaline. The structural relationship of penicillin to glutathione has received some attention (68), but addition of glutathione to growing cultures has had no effect on penicillin production.

Biosynthesis of possible intermediates by cultures not producing the intact penicillin molecule has been studied without success. This has been approached by growing mixed cultures of nonproducers and producers (19), and by adding culture fluid from penicillin-producing cultures to these freshly inoculated fermentations (38, 39). A number of biochemical mutants of penicillin-producing cultures which require certain amino acids or growth factors for growth have been studied to determine whether these growth requirements influence the production of penicillin by these cultures (18, 19). These studies have not shown any correlation between a specific requirement and penicillin production.

SUMMARY

During the past ten years a multi-million dollar industry has developed, based on the mycological production of penicillin. Studies on factors influencing penicillin production have shown that selection of cultures, formulation of media, addition of intermediates in penicillin biosynthesis to the fermentations, and selection of optimum aeration and fermentation conditions play a part in the successful operation of this fermentation process. Although many studies have been made on the chemical changes occurring during the growth of the fungi and production of penicillin, little information has been gained on the mechanism of penicillin formation by fungi. However, these studies have helped in formulation of synthetic media which support growth and penicillin production equivalent to those obtained with media containing natural material.

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THE BOTANICAL REVIEW

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CHLOROPHYLL

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Iowa State College

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INTRODUCTION

The purpose of this review is to summarize certain areas of existing information concerning the chlorophylls. No attempt has been made to include all publications relevant to these substances, and a number which are mentioned have been available only as abstracts. The latter will generally be acknowledged as such. Certain phases have been largely omitted, e.g., photosynthesis (especially kinetic studies of gas exchange, the path of carbon, and to some extent physico-chemical studies of fluorescence in living tissue), phototaxis and photoperiodism. These are too extensive as such, and only those parts of them are covered as are directly concerned with chlorophyll. Summaries of certain other phases of chlorophyll are available elsewhere, e.g., the chemistry of chlorophyll (a variety of reviews is available); analytical methods, their medicinal and industrial use (13*a*); and absorption spectra of chlorophyll and related compounds (13*a*).

---

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TYPES

There have been described, at present, the following chlorophylls: *a, b, c, d,* bacteriochlorophyll (*a, b* and *c*) and protochlorophyll (possibly *a* and *b*). Of these at least the first two may be isomerized to forms termed *a′* and *b′*. A material previously isolated from the pigments of higher green plants (241) and then termed "chlorophyll *c*" was later found (135, 242) to be an artifact. Of the three photosynthetic groups—green plants, purple sulfur bacteria, and green sulfur bacteria (157)—the photosynthetic pigment in the last group is not yet precisely known. A table by Cook (38) of the chlorophylls in different algae is given in Table I.

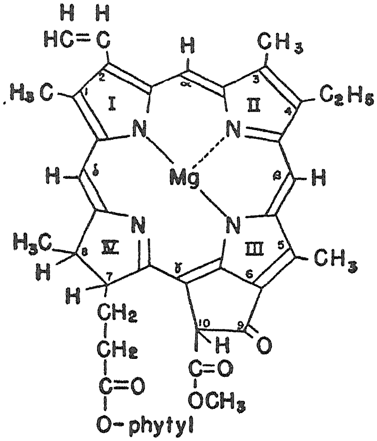
TABLE I

KNOWN DISTRIBUTION OF CHLOROPHYLLS IN ALGAE (FROM A. H. COOK)

	a	*b*	*c*	*d*
Chlorophyceae	+	+		
Xanthophyceae	+			
Chrysophayceae				
Bacillariophyceae (diatoms)	+	+	+	
Dinophyceae (dinoflagellates)	+		+	
Euglenineae	+	+		
Phaeophyceae	+		+	
Rhodophyceae	+			+
Myxophyceae	+			

The formulae and properties given for chlorophyll are of necessity those of isolated material. The extent to which they differ from the material as present in the cell is not adequately known; the nature of the difficulties will be indicated.

CHLOROPHYLLS *a* AND *b*. The material which is the predominant coloring of living matter on the surface of the earth is chlorophyll *a*. Its formula (I) differs from that of the generally accompanying chlorophyll *b* (II), as has long been known, by possessing a methyl instead of a formyl group in the 3-position. The normal approximate ratio of these pigments in higher plants is 3/1 (*a/b*). Their absorption spectra (Fig. 1) are now known with sufficient precision for all analytical work (134, 243).



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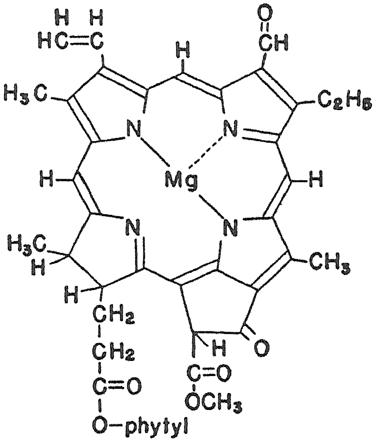

II

Seybold, Egle and Hulsbruch (196, 197) have extended their investigations for the chlorophyll contents of fresh-water algae and have come to the general conclusion that pigment investigations provide a sound basis for the examination of phylogenetic relationships in algae. Gessner (72) has indicated that the chlorophyll content of the plankton from Bavarian lakes provides a good measure of the production capacity of the corresponding water, a conclusion more or less previously reached by Manning and Juday (136) for Wisconsin lakes. Dutton and Juday (47) were unable to correlate chromatic adaptation of fresh-water plankton with depth distribution. The change in Palogloea from yellow in the upper zones to bright green in the 4–10 m. zone, which was the result of chlorophyll increase, carotenoid remaining constant, is explained as probably being due to a response to low light intensity rather than being that of a chromatic adaptation.

Inman (108) found that thermal algae (Myxophyceae) and *Chlorella* growing in temperatures of 37–72° C. had normal chlorophylls and photosynthesized not only at the growth temperatures but at colder values.

It is interesting to note that the parasitic mistletoe, *Phoradendron flavescens,* contains chlorophylls *a* and *b* and also photosynthesizes (68). The nature of the deficiency is, therefore, of interest. Presumably it is the inability of this plant to perform its root functions, i.e., obtain water and nutrients.

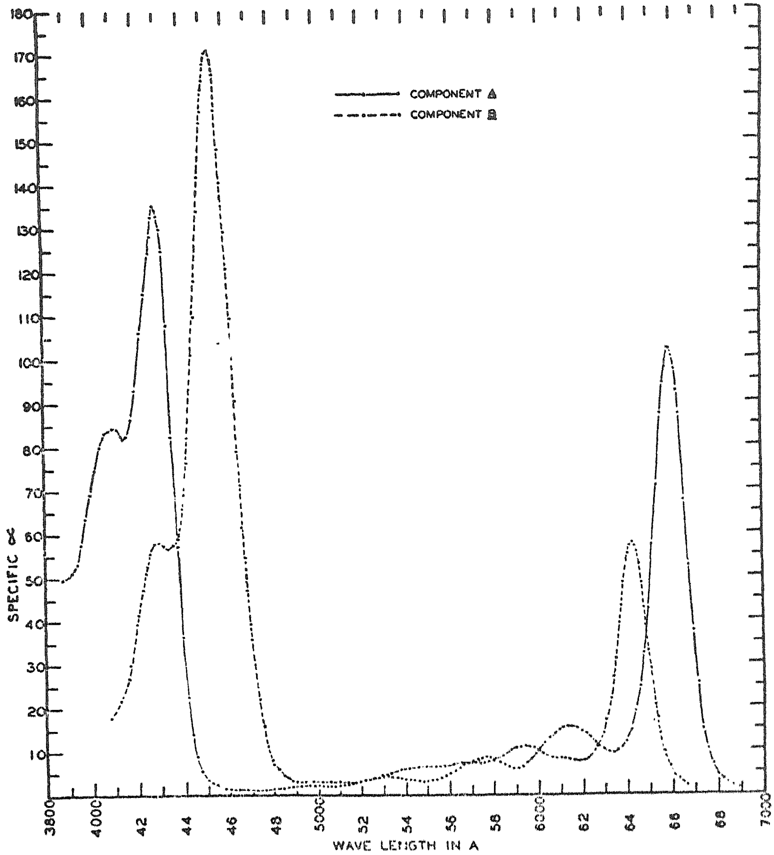

Fig. 1. The absorption spectra of chlorophylls *a* and *b* (from Zscheile and Comar (243)).

Of predominate interest in chlorophylls are the locations of hydrogens which may be active in photoreduction. The activity of the hydrogen on C–10 is well known. Among the more spectacular demonstrations of this is the keto-enol tautomerism, shown by the formation of a benzoyl derivative of the enol (219) and implied in the spectrum of phylloerythrin (13). To this carbon has also been ascribed the properties involved in the reversible leuco formation of chlorophyll (125) and various photochemical studies of chlorophyll discussed elsewhere. Furthermore, because of the ease of isomerism of the pyrrolenin hydrogens (on 7,8) saturating the vinyl group and forming porphines, it is conceivable that these hydrogens may also be involved in photochemical reductions. A model reaction of this type is available in the photochemical oxidation of the Zn salt of the synthetic tetraphenyl chlorin of the corresponding porphine (15, 35).

Strain and Manning (220) have provided evidence of an isomerization of chlorophylls *a* and *b*, the isomers being termed, correspondingly, *a′* and *b′*. The isomers, in *n*-propanol, are interconvertible slowly at room temperature, rapidly at 100° (equilibrium mixture 4 *a* or *b* / 1 *a′* or *b′*). The isomers yield spectrally different pheophytins but similar pheopurpurins. They are made evident by chromatography upon dried confectioners' sugar, using petroleum ether–0.5% *n*-propanol–0.5% purified dimethylaniline as wash solvent. The *a′* appears below the *a*, the *b′* below the *b*. No explanation as to character of the isomerization is offered, except that, since different pheophytins are obtainable, the character of the Mg bonding is not involved. According to the present formula of chlorophyll, it is possible that isolation of the keto-enol tautomers have been accomplished, or even the cis-trans isomers originating from the positions of the hydrogens at 7,8. An additional possibility[2] is that of the separation of the chlorophylls into the geometrical isomers corresponding to the cis and trans forms in the phytol.

Seybold (189, 191), correlating the chemical compositions of sun- and shade-loving plants with that of the various algae which lack chlorophyll *b*, has come to the conclusion that the chlorophylls have different functions aside from that of photosynthesis. To chlorophyll *a* and its protein moiety he ascribes the formation of glucose and other monosaccharides, while chlorophyll *b* polymerizes the monosaccharides to form starch. This theory arises from the absence of *b* in those algae which do not form starch, and its presence in those which do. Why a similar variation does not occur in, e.g., young wheat plants, which do not form starch, and old ones, which do, is not considered.

CHLOROPHYLL *c* (chlorofucine, chlorophyll γ, chlorophyllin γ). This pigment, noted as long ago as 1873 by Sorby (215) and believed to be an artifact by Willstatter and Page (233), though denied by Wilschke (232), has been thoroughly confirmed in investigations by Strain *et al.* (221). Using centrifuged pure cultures of diatoms and algae, extraction was accomplished with absolute methanol containing 0.2% dimethylaniline, which, after addition of water, was partitioned with petroleum ether, the xanthophylls and chlorophyll *c* remaining in the methanol layer. On further addition of water, additional contaminating pigment could be transferred to ether. With addition of fresh ether and excess salt solution to the aqueous layer the chlorofucine could be transferred to ether. A simpler method of preparation involves transfer of the total pigment complex to ether, concentration of the latter and its dilution with petroleum ether, followed by chromatography on powdered confectioners' sugar, the chromatogram being developed with petroleum ether–2–4% methanol. The chlorophyll *c* is described as forming a pale green band at the top of the column, underlain by fucoxanthin, while other materials, as chlorophyll *a*, and carotene pass through rapidly. The pigment prepared by the adsorption method possesses a spectrum somewhat different from that given for the partition method (Fig. 2), though the latter could be made to yield spectra more like the former by chromatography. It therefore appears that chlorophyll *c* either is still a mixture or is subject to changes, as oxidation or isomerization, on chromatography. More specifically, chlorophyll *c* is not equivalent to chlorophyll *a'*.

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[2] Manning, W. M. Personal communication.

Granick (84, 85) has recently shown that chlorophyll *c* possesses Mg (removable by 4 *N* HCl), gives a phase test, lacks phytol and, following Mg removal, gives a porphyrin spectrum. He thus suggests that chlorophyll *c* is a Mg pheoporphyrin, more closely allied to protoporphyrin than to chlorophyll itself.

CHLOROPHYLL *d*. Using a procedure essentially similar to that given above, but taking advantage of greater ease of extraction, a new green pigment, chlorophyll *d*, has been found in red algae by Manning and Strain (137). Finding no chlorophyll *b* or *c* in these algae, separation was most readily effected after partial extraction by chromatography on powdered confectioners' sugar. The chlorophyll *d*, which is found above chlorophyll *a*, yields the spectrum given in Fig. 3. (The prominent band ca. 460 mμ would indicate a contaminant of some kind, especially since this is missing in the corresponding pheophytins; this possibility is not indicated by the authors.) After ashing of a specimen, the metal in the center was shown colorimetrically by Titan yellow to be Mg.

As in the case of chlorophylls *a* and *b*, chlorophyll *d* is also found to isomerize in a bewildering number of manners. The pigment in methanol is converted into three other compounds, rapidly on heating, slowly at room temperature. One of the three has a spectrum similar to that of chlorophyll *d* and is termed *d'*. The other two (all three being evident chromatographically) have different spectra, though resembling each other, and are termed isochlorophylls *d* and *d'*. The *d*'s and *d'*'s are interconvertible in the same manner as the *a*'s and *a'*'s. Furthermore, although by indirect evidence only, it is believed that isochlorophyll is interconvertible

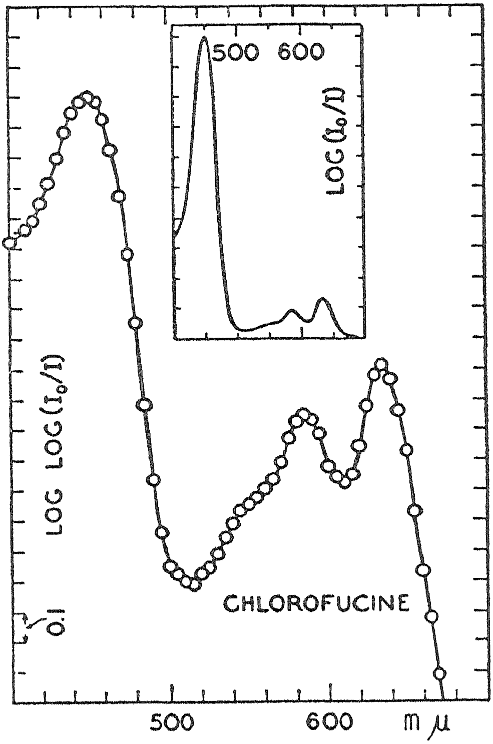

FIG. 2. The absorption spectrum of chlorophyll *c* (from Strain, Manning and Hardin (221)).

with chlorophyll *d*. Addition of Mg grignard to a dry ethereal solution of isopheophytin *d* resulted primarily in isochlorophyll *d*, but it is stated that both *d'* and *d* were produced by isomerization of this synthetic isochlorophyll *d*. On the other hand, pheophytin *d* did not yield primarily chlorophyll *d* or the iso-compound, but a mixture of unknown materials. The type of pheophytin formed appears to be a rate phenomenon, the compound produced at −80° C. being pheophytin *d*; at room temperatures, isopheophytin *d*. Furthermore, at room temperature, by addition of acid, pheophytin *d* is rapidly converted to the iso-derivative. The iso-series thus appears to be the more "stable" series and to have spectral and adsorption properties similar to those of chlorophyll *a*. It is apparent that some organic chemistry remains to be done in the chlorophylls.

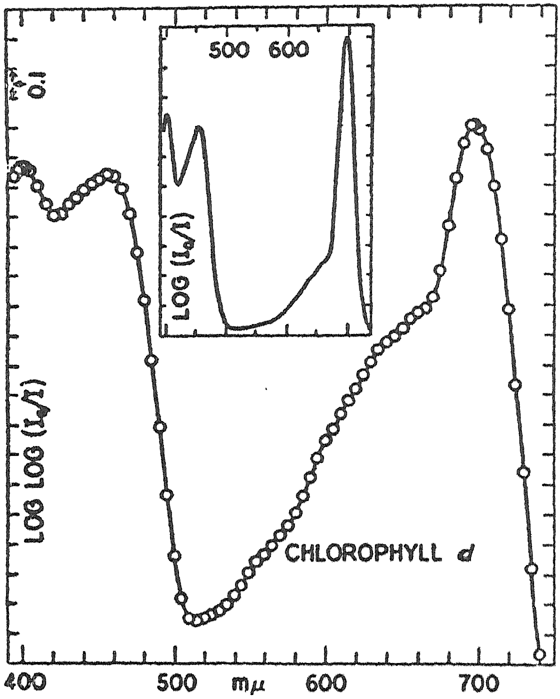

FIG. 3. The absorption spectrum of chlorophyll *d* (from Manning and Strain (137)).

BACTERIOCHLOROPHYLLS. Bacteriochlorophyll is 3,4 dihydrochlorophyll *a*. Its preparation involves difficulties, primarily of oxidation, since extreme care must be used to avoid removal of two of the four hydrogens which form pyrrolenin rings. In a continuation of earlier work, Mittenzwei (144) has substantiated the previous tentative assignment of the additional two hydrogens in the 3,4 positions. The bacteriochlorophyll, isolated on the oxidized product 2 acetyl (2-desvinyl) chlorophyll *a*, could be reduced to the 2,α-oxy-meso derivative[3] and thence, by heating in a high vacuum, to the 2-vinyl, i.e., to chlorophyll *a* itself. The assignment of the two additional labile hydrogens was based on oxidations with chromic acid, from the basic fraction of which an optically active oil, α-methyl α'-ethyl succinic anhydride, was obtained. This could have come only from ring II or III and, because of the identity produced in the conversion of bacteriochlorophyll to chlorophyll *a*, is ascribed to ring II. Seybold and Egle (193) have treated *Thiocytis* bacteria in a manner similar to Strain *et al.* and have come to the conclusion that there is but one bacteriochlorophyll *a*, but that two others, *b* and *c*, are artifacts resulting from the method of preparation. The methanol extract of the bacteria was transferred to benzene to the extent to which pigment would enter. The solution was then concentrated under inert gas and the mixture chromatographed on powdered sucrose, resulting in a green bacteriochlorophyll *a* on top and a blue *a* below it. By salting the methanol solution, further pigment was forced into additional benzene, and a third pigment, bacteriochlorophyll *c*, obtained. Chlorophylls *b* and *c* had relatively similar spectra, with the main band in the red at 695–665 mμ, while that for *a* lay at 700–665 mμ, indicating a somewhat further maximum into the infra red. If the original bacterial solution was converted directly into pheophytin by extraction with methanol containing oxalic acid, only the pheophytin of *a* was obtained. If, however, a solution of pure *a* was allowed to stand in methanol for several hours and then converted to pheophytin, both *a* and *b* were obtained. It is thus presumed that bacteriochlorophylls *b* and *c* are artifacts. The close resemblance of these reactions to the isomerizations of Strain and Manning are obvious.

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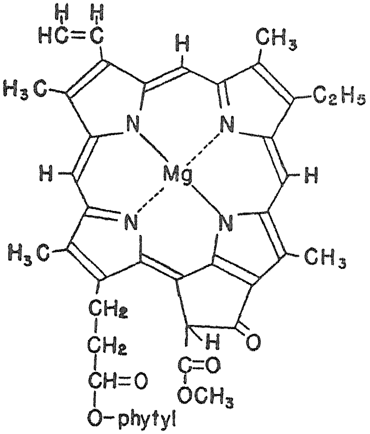
[3] Meso-compounds are those which contain two additional hydrogens, generally a saturated vinyl group. Meso-chlorins and phorbins thus possess the two hydrogens at 7 and 8, but contain a 2-ethyl rather than a 2-vinyl.

PROTOCHLOROPHYLL. The porphine nature of protochlorophyll (III) was first shown by Noack and Kiessling (161), and its definitive structure as the Mg chelate of the phytol-esterified vinyl pheoporphine a_5 (61, 62) by Stoll and Wiedemann (219). The former workers also suggested that protochlorophyll is a precursor, rather than a degradation product, of chlorophyll. Seybold and Egle (192) are of the opinion that there is a protochlorophyll *b* as well as the corresponding protopheophytins. On the basis of spectral comparison with synthetic protochlorophyll this has been questioned by Fischer and Oestreicher (*loc. cit.*), as in the protochlorophyll (Fig. 4) isolated by Koski and Smith (118) from etiolated barley seedlings. The protopheophytins are probably artifacts of preparation, although they, too, were noted in the work by Koski and Smith. These authors also confirm the action spectrum of chlorophyll formation (67) which was the first positive proof of the origin of chlorophyll from protochlorophyll. At that time the action spectrum was somewhat at odds with the absorption spectral data of Seybold and Egle, but the latter are now known to be inaccurate.



III

THE NATURAL STATE

One of the most persistent problems in plant physiology is the nature of the existence of chlorophyll, whether it is bound to a

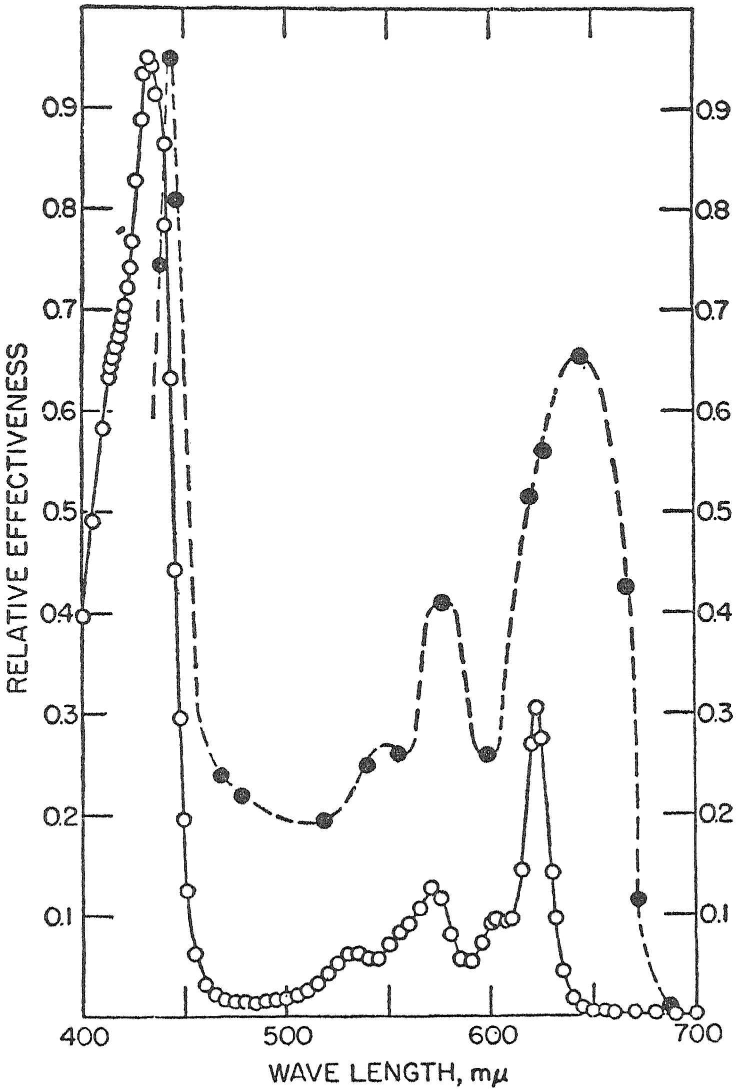


FIG. 4. A comparison of the action spectrum of chlorophyll formation (dotted lines) and the absorption spectrum of protochlorophyll (solid line) (from Smith (214)).

protein, whether it is present as a solid-liquid colloid, or dissolved in an oily matrix, or linked in a complex manner with a variety of substances.

It is of interest to summarize an analysis of a typical chloroplast. Menke (140) has shown that leaves average 17% chloroplasts. Using differential centrifugation (139, 83), he obtained essentially pure chloroplasts, analysis of the material showing it to be 47.7% lipid, 37.4% protein, 7.8% ash, 7.1% undetermined. Chibnall (36) gave as the analysis of the lipids of typical chloroplasts of cabbage (% of total ether extract): chlorophylls, 9.3%; carotene, 0.5%; xanthophyll, 0.8%; glyceride fatty acids, 17.5%; wax, 12.3%; sterols, 4.5%; undetermined unsaponifiable material, 13.3%; calcium phosphatide, 18.4%. It is thus apparent that the primary matter of chloroplasts is protein and that considerable matter is there in greater quantity than that of chlorophyll. Analyses similar to the above are also given by Bot (28). Hanson *et al.* (94) state that in young leaves chloroplast protein is only about 7% of the total protein but later increases to 35–40%. The chloroplast proteins are exceptionally rich in S, containing approximately 70% of the total protein S of the leaves.

Godnev and Kalishevich (77), among others, have investigated the concentration of chlorophyll per unit volume of tissue, using the chloroplasts of *Mnium medium* which possesses very small leaves. The average number of chloroplasts per leaf was 775,000. The total chlorophyll per leaf was (for 22 leaves) 1.91 γ. The average amount of chlorophyll per chloroplast was, therefore, $2.4 \cdot 10^{-6}\ \gamma$. We may extend the data of these authors further, using 4 μ as an average diameter for the chloroplasts, which then have a volume of $35\ \mu^3 = 35 \cdot 10^{-12}$ cm.^{3} In this volume then are the $2.4 \cdot 10^{-6}\ \gamma$ chlorophyll, which is equivalent to a concentration of $2.4 \cdot 10^{6}\ \gamma/35$ cm.$^3 = 72 \cdot 10^{6}\ \gamma$/liter $= 72$ g./l. This is, then, a molar concentration of $72/890 = 0.08$ or approximately 0.1 *M*. Calculations made with other plants by other authors [see the discussion of the above subject in Rabinowitch (180)] have given values as high as 0.2 *M*. Consider the spatial significance of such a concentration, assuming uniform distribution and neglecting, for the moment, the requirements of the phytol, concerning ourselves solely with the phorbin nucleus. On the basis of X-ray studies of the phthalocyanines, and our general knowledge of porphyrin structure, we may safely assume an average diameter for the phorbin ring as somewhat greater than 10 Å. An isotropic distribution for the $12 \cdot 10^{22}$ molecules/liter in a 0.2 M solution results in a unit volume/molecule of 8 $Å^3$ (i.e., there are $1.2 \cdot 10^{23}$ molecules in 10^{24} $Å^3$). Obviously the concentration of 0.2 M is possible only if considerable packing occurs, so that the average distance between planes is less than that of their diameter. However, the phytol, which has heretofore been neglected, must assume a position between phorbin planes. It would thus appear that even a 0.1 M concentration of chlorophyll in the chloroplasts is an almost saturated solution. Inasmuch as the chlorophyll represents only a minor portion of the chemical species, the chloroplasts are thus depicted as highly concentrated bodies, both the chemistry and physics of which are probably not deducible directly from observations on more dilute solutions. Under these conditions spectral observations, energy transmission, etc., assume different aspects than normal. Thus, although one of the common arguments in favor of the colloidal state of chlorophyll is the red-shifted spectrum of chlorophyll in leaves as compared to its solution in organic solvents, a condition which can be duplicated by synthetic colloidal preparations, it is well known that concentrated substances may often polymerize somewhat, resulting in spectral shifts. To what extent this accounts for leaf spectra is not known, and recent studies in this regard (Fig. 5) (107, 141, 179, 198) still do not take this point of view. Meyer, however, also believes that the

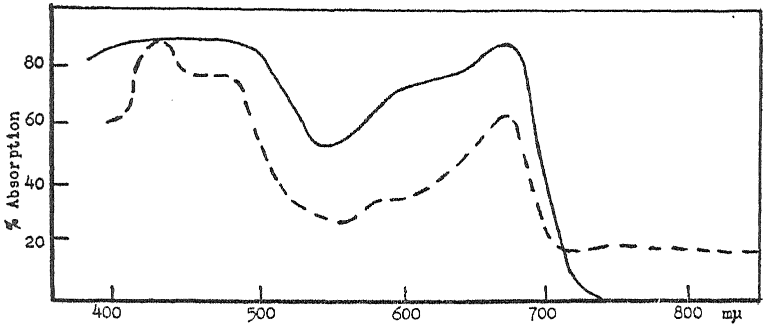


FIG. 5. Absorption spectrum of *Dolichos* leaf (solid line) and a suspension of chloroplasts from a *Dolichos* leaf (dotted lines) (adapted from Rabideau, French, and Holt (179)).

chlorophyll extracted from leaves by present methods yields a material which is not identical with that present as native chlorophyll, although his method, which does not differ radically from present procedures, does. Both Bot (28) and Seybold and Egle (194) have suggested that chlorophyll is present in leaves as two phases, one dissolved in lipids and the other bound to protein. Synthetic mixtures of this type have recently been made by Lepeschkin (124), which exhibit staining and solubility properties similar to those of chloroplasts. Following the suggestions of chlorophyll-protein combination by Lubimenko (131), Noack (159) succeeded in preparing material containing both precipitable protein and chlorophyll, and, more important, chlorophyll which fluoresced, a characteristic of undenatured (non-precipitated) material. Further, by various conditions which denatured the protein, the fluorescence of the chlorophyll disappeared, indicating that the chlorophyll was flocculated. There have been similar recent studies (3, 63, 64, 68, 149, 208–212) aside from those of Menke.

Fishman and Moyer have concerned themselves primarily with the electrophoretic-pH curves of various legumes and of *Aspidistra*. The material isolated from legumes, undoubtedly of a nature akin to the "grana" used by a number of workers, and probably containing by adsorption or combination a host of other substances besides chlorophyll and protein, showed an isoelectric region near the pH of 4.7 (0.02 *M* acetate buffer, 25°). The migration rate was independent of size, indicating that ionic charges, rather than zeta potential, were the predominating cause for electrophoretic migration. From the nature of the curves it was suggested that the proteins of the different legumes were all similar. In this regard some interesting experiments (116) were performed on colloidal chlorophyll in water which was shown to be negatively charged in a Zn-Cu couple. In a quartz cell with Pt electrodes, the particles, observed with an ultramicroscope, went to the anode, and reversing the current reversed the movement. With a potential difference of 1 v./cm. their speed was $30.3 \cdot 10^{-5}$ cm./sec.

The results, therefore, indicate particles of large state of aggregation. Bearing a negative charge, the particle could be flocculated by cations; the relative amounts of KCl, $MgCl_2$ and $AlCl_3$ required for flocculation were $2000 \cdot 100 \cdot 1$.

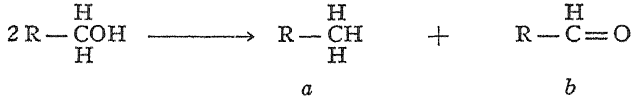
Studies on chlorophyll–protein relationship have been made with the aid of detergents, briefly by Kuhn *et al.* (122) and rather extensively by Smith (*loc. cit.*) and Smith and Pickels (*loc. cit.*). The primary visible effect of the detergents is to clarify the opalescent solutions. In the case of dodecyl sulfate, this also removes the Mg from the chlorophyll, converting it to pheophytin. Since, however, the chlorophyll-protein bonds remain, it is concluded that the complex is not bound through Mg. Pheophytin formation, which occurs in the above even at the pH range of 8–9, does not occur with digitonin or bile salts at pH 4–5.

EFFECT OF RADIATION ON CHLOROPHYLL IN THE PLANT

There will be discussed in this section the effect of light on plants as a requirement for chlorophyll development, and destructive effects.

It is well known that light is required for the development of most, though not all, chloroplasts. Some algae, e.g., when fed a proper source of food, as glucose, can develop normally in the dark, producing chlorophyll as if they were normally growing in light. On illumination the capacity for photosynthesis generally does not appear to be inhibited. In the lotus embryo, Stewart and Eyster (218) report only chlorophyll *b*, with no *a* (see also Seybold and Egle (195) on pigments of spores and seeds). The majority of plants, however, require light not only for photosynthesis but for the very development of chlorophyll. The most direct method of investigation of the role of light is to determine its action spectrum, i.e., the effectiveness of the different wave lengths in producing chlorophyll. Using Corning filters in various combinations to give bands with a half-width averaging ca. 40 $m\mu$, the effectiveness of the spectrum has been evaluated by Frank (67), using the amount of chlorophyll formation as a measure of the effectiveness. Under the conditions involved there was also carotenoid development, which was particularly prominent in the young *Avena* plants used and which affected the amount of incident light available to the plants. It was found, however, that the ratio of the effectiveness in chlorophyll formation of the blue light, which the carotenoids absorb, to that of red light, which they do not, did not change with age of the young plants (72, 96 and 120 hours old), despite a known doubling of the carotenoid concentration during that period. The conclusion was thus reached that the available light was not screened out by the carotenoids, which must thus be located morphologically behind the light-absorbing active substance in the chloroplast. The action spectrum thus obtained (corrected for the spread of the filters) is given in Fig. 4. The spectrum resembles that for protochlorophyll relatively well. A partial explanation of the earlier difficulties may also be found in Seybold's (1939) (*loc. cit.*) reporting of the almost constant occurrence of protopheophytin along with protochlorophyll, also called "trichosanthin". The width of the band in the red, which does not occur to the extent found in porphines, must thus be attributed to an accompanying material; that is, at least two pigments are involved. This is made even more probable by the narrowness of the spectrum in the blue, where the bands of the two pigments would be expected to coincide. It is indeed possible that there are protochlorophylls corresponding to the separate chlorophylls *a* and *b*. Results consistent with the above have been given by Guerrini (88) with more monochromatic light. The contention of Barrenscheen *et al.* (16) that etiolated wheat seedlings do not possess protochlorophyll because their red-fluorescent ether extract did not possess a visible absorption spectrum, is unwarranted in view of the sensitivity of the fluorescence, which can determine porphyrin content 1/10 as much as the limit of visible absorption spectra. Ultraviolet light, however, poses a different picture, since protein may become denatured by its action. Although Eilert and Giersch (50) did not note any effect of the filtrate of UV-irradiated *Stichococcus* on the chlorophyll or carbohydrate content of algae (there is no indication of the extent of absorption by the algae of the materials), there is an abundance of evidence to show the debilitating action of the UV rays themselves. Thus Gilles (74) notes that, although plants containing chlorophyll are more resistant to UV rays than those not having it, since the chlorophyll obviously absorbs much of the radiation until it is itself destroyed and photooxidized, the wave lengths most effective in destruction are 290–310 mμ, where proteins as well as a variety of other substances are particularly effective absorbers. Montfort (146) emphasizes that the photooxidative destruction does not commence until photosynthesis has decreased to a small fraction of its nominal value. With Zöllner (148) he concludes that even strong light is not always harmful, as is the case in Nympheae.

Goodwin and Owens (80) report that on exposure to light (4358 Å) young oat plants form chlorophyll *a* but no chlorophyll *b* in three hours. The ratio of chlorophyll formation from protochlorophyll has been beautifully studied by J. H. C. Smith (214). It is shown that in etiolated barley seedlings the ratios of formation of chlorophyll *a* to *b* remain constant under a variety of temperatures and in both constant and intermittent illumination after a short period in which only chlorophyll *a* is produced. The suggestion is thus made, although it does not appear obvious, that chlorophyll *b* is not produced from *a*. The argument advanced is that if it were produced photochemically the ratio of formation would not be constant but continuously increasing. Since the discussion concerns ratios, not absolute amounts, it does not seem improbable that a definite fraction of the *a* is transformed photochemically to the *b*. That the ratio remains constant and similar during intermittent illumination as compared with constant illumination is the basis for indicating that *b* is not formed by *a* by a thermochemical reaction. This argument is faulty inasmuch as more *a* might well be produced in intermittent than in continuous illumination. If the amount of *b* formed is strictly a function of the amount of *a* present, then the above conditions do not invalidate the formation of *b* from *a*. The conclusion reached by the author and his associate is that chlorophylls *b* and *a* are formed from a common precursor by disproportionation, i.e.,



$$2R-\overset{H}{\underset{H}{C}}OH \longrightarrow \underset{a}{R-\overset{H}{\underset{H}{C}}H} + \underset{b}{R-\overset{H}{C}=O}$$

Other work by the same author (214) has shown that the amount of chlorophyll formed is directly proportional to the amount of protochlorophyll present in the leaf. Furthermore, by controlling the temperature and thus the rate of dark formation of protochlorophyll, he also controls the subsequent amount of chlorophyll formation. Biebel has reported (22), in work probably best interpreted as illustrating photoperiodism, that chlorophyll formation of kidney-bean seedlings increased with increasing frequency of light cycling up to 144 times per day.

The only recent report dealing with the effect of materials developed or used in radioactive investigation is that concerning the effect of neutrons on photosynthesis in *Scenedesmus* and *Chlorella.* Nisina, Nakamura and Nakayama (158) irradiated these algae with fast neutrons from a deuteron (3 mev, 20 microamp.) bombardment of beryllium. A short irradiation of *Scenedesmus* (10′) had no effect on photosynthesis, while somewhat longer actually increased it, no inhibition being observed until after 50′ (36% diminution after two hours). In *Chlorella,* after one hour, a 46% diminution had occurred. Respiration in *Scenedesmus* was not affected by the 3-hour exposure.

CHLOROPHYLL IN RELATION TO PLANT COMPOSITION AND NUTRITION

Physiological studies often give determinations of chlorophyll along with those of numerous other constituents, e.g., potassium, nitrogen, iron, carbohydrates, ascorbic acid and organic acids. Occasionally conclusions are drawn regarding the relation between changes in the environment and their effect upon the isolated systems, as determined by analysis. It is obvious that in an organism in which the various portions are strictly dependent upon each other, anything which influences one will influence another. This is true not only in the larger sense but also in the smaller, i.e., with respect to the deficiency of a trace element, which affects, e.g., those of amino acid formation, will obviously affect protein formation. Disturbance of the protein will in turn affect all enzymatic reactions, since there are probably few if any of the latter which do not have a protein as a carrier.

It is possible, however, to gain somewhat of an insight into the relationship of reactions by a study of the rates when one of the factors is limiting. Plant physiological studies have not progressed in this direction sufficiently, however, and most problems have been attacked in a cause and effect manner. One of the better-known problems is that of the relationship between iron and chlorosis. Every student knows that the leaves of growing plants may be made chlorotic by a withholding of iron from the system. It has, therefore, been concluded that iron, per se, is involved in chlorophyll formation, and functions have been assigned to iron with evidence therefor (see section on theory of formation). Recent work on the nature of iron metabolism has been reviewed by Bennett (20). A variety of interesting relationships have been shown for chlorotic pineapple (202, 204) fed iron and either ammonium ion or nitrate, not so much in the expected higher sugar yields but in variations of intermediates, as ascorbic and other organic acids. This would depend on the rate of nitrate reduction as compared to the rates of ascorbic formation as well as utilization. The ascorbic acid and other organic acids are decreased in the nitrate series; are they, therefore, involved in nitrate reduction? A similar but less ambitious study on sugar and N metabolism has been given by Cooke (39). More specific, however, is the work by Hill and Lehmann (97). These workers realize that the primary difficulty in analysis lies in the interpretation of the analytical data, i.e., of the various "classes" of iron resulting from different methods of determination. Thus there arose the concept of "active iron" by Oserkowsky (165). A linear relationship between chlorophyll and Fe was found when the active iron was plotted against the amount of chlorophyll. In this work the "active" iron was that extractable by dilute hydrochloric acid. Hill and Lehmann divide the water-soluble Fe into three categories: *a*) that which reacts with α, α'-dipridyl immediately; *b*) that which reacts only after boiling in acid; and *c*) that which reacts only after ashing. The Fe/chlorophyll ratio, varying from 1/4 to 1/10, also fluctuated with time, since the Fe in the chloroplasts preceded chlorophyll both in appearance and in disappearance. Jacobson (111) showed that if the leaves of the material were properly washed to remove surface iron, a proportionality could be found between total Fe and chlorophyll. However, before chlorophyll formation could occur, the iron in the chloroplasts had to exceed a threshold, indicating the prior necessity of other reactions for iron. A similar situation is known to exist for Mg. Thus, although a linear relationship was found to occur with the "active" iron and the chlorophyll, it also existed with other components, e.g., the protein precipitable by ammonium sulphate. Indeed the data are interpreted by Bennett (*loc. cit.*) as that of an association of the active iron with the active protein which is in turn responsible for chlorophyll formation. Thus it appears that the well-known iron-chlorosis phenomenon is more closely concerned with protein formation rather than chlorophyll formation. (See also 201 for analogous conclusions based on nitrogen studies.)

Studies in a similar light (e.g., 184) indicate that K "facilitates" transfer of Fe and thus influences chlorophyll formation. The difficulties involved are further illustrated when careful workers as Sideris and Young (203) state that in pineapples, at least, chlorophyll or carotenoids are not affected by the K level of the cultures. Schropp (188) says that, although the effects of B on chlorophyll formation are variable, those of Cu, Mn and Zn definitely indicate chloroses in the event of deficiency. The necessity of these elements for various enzymatic reactions is becoming increasingly evident; e.g., witness the ability of Zn and Cu to relieve the inhibition by phenanthroline of the photochemical reduction of quinone by chloroplast grana (see photochemistry sections).

A direct approach to the effect of Mg deficiency has been given by Kennedy (115) in which the deficiency is studied not only as a function of the chlorophyll concentration but also as a function of the rate of photosynthesis per unit of chlorophyll per flash. It was shown that Mg-deficient *Chlorella*, in contrast to Fe-deficient, affected the rate of the "dark reaction", i.e., the amount of photosynthesis per unit flash was decreased if the dark period was not of sufficient length, which did not occur in Fe-deficiency. However, it is well known that Mg fed to deficient plants is localized in other compounds previous to chlorophyll [see, e.g., Smith (1947) in section on theory of formation and Jevillier and Goodchauz (112)], and it is therefore not possible to conclude from the above that chlorophyll has a dark function as well as a light function.

A host of papers exist on various factors and their relation to chlorophyll development or function. It is impossible to assess all, and a few are selected for illustrative purposes. A study has been made by Kohler (117), for example, of the composition of oats at various stages of growth. The factors analyzed included a number of the enzymes as well as fat, fiber, chlorophyll, protein, etc. The majority of the factors analyzed showed maximal amounts at the jointing stage. More or less routine determinations were made by other authors on pigment concentrations in fresh and processed Swiss chard and beet greens (176); on pigments in a variety of tobacco (86); and on assorted items, including pigments, of summer squashes (102) and of beet leaves (163). Soil studies include those (205) in which the effect of various types of fertilizers are studied by noting the rate of photosynthesis of the plants, although it is difficult to understand why this is termed equivalent to the determination of chlorophyll, which would appear both easier and more convenient. A study has been made by Powers (177) of boron, which includes significant increase in chlorophyll (50%) after addition of B to soils deficient in that element.

Beck (19) studied the effect of drought on pigments of sunflowers, the previously dried soil being saturated with aqueous sugar solutions of various concentrations. It might possibly have been more advisable to regulate the water content with an inert carrier rather than introduce another variable. However, the major factors found to influence pigment production were not so much drought, this being effective only under extreme circumstances, but light and heat. Zaitseva (237) has found a temporary pigment effect after vernalization on wheat seedlings, the first leaves of vernalized seedlings showing more chlorophyll than non-vernalized seedlings.

Studies of the pigment content of algae continue. Graham (82) has published a generalized study of the chlorophyll content of plankton, showing that if due account is taken of the size and chlorophyll content of different species of plankton life, the chlorophyll content of plankton can be taken as an indication of the photosynthetic capacity of sea water. More specific studies for different groups of algae have been made by Seybold *et al.* (196). Skopintsev and Bruk (207) have studied the rate of decomposition of chlorophyll and phosphorus compounds of dead plankton. At 16° C., 82% of the chlorophyll is destroyed within 53 days, a condition similar to that for the P-compounds but much slower than that required for ammonification.

Spoehr and Milner (216), in describing the variation of *Chlorella* composition with differing inorganic media, noted that under conditions of low nitrogen the metabolism is shifted toward lipid synthesis. Comparison of cultures of increasing lipid content showed a decrease in chlorophyll content (down to 0.012% which is equivalent to 1/500–1/2000 of those of low lipid content), following which no further decrease occurred, though the cells continued to grow. The carotenoid decrease accompanying the chlorophyll change is only one-tenth the magnitude. The experiments are noteworthy in their exemplification that the composition of a unicellular plant is not a constant, even within narrow limits, but can be made to vary with the environment. It is probable that the greater the number of cellular types (tissues) dependent upon each other, the less the allowable variation in composition. A similar study on NO_3^- and NH_4^+, less extensive and designed primarily for photosynthetic investigation, has been given by Myers (153).

The problem of the daily variation of the chlorophyll content continues. Using chromatographic technique, Wendel (230) made determinations of chlorophyll on three- to four-hour bases, finding a definite maximum at noon for four different plants. These variations were not found in darkened plants. Bukatsch (31), however, found the minimum weight of *Adenostyles* leaves to occur at the same time as their chlorophyll minimum, indicating a possible source of the variation. This is another of the type of problem which is vexatious and laborious by ordinary means and is readily solved with the use of isotopes, since all that needs to be done is to let a plant photosynthesize with isotopic CO_2 long enough to develop a certain amount of chlorophyll. Thereafter one need merely determine the specific activity of the chlorophyll at desired intervals.

An interesting genetic approach to chlorophyll formation, aside from that of the albinos previously mentioned, is that offered by Iuracec (110), in which it is noted that the chlorophyll content of fresh female and male plants of *Urtica dioica* are not equal but exist in the ratio of 1 : 1.15 (range 230–296 : 189–277). Obviously the first need is for more uniform tissue, following which cytological studies should be correlated with biochemistry. Miller and Johnson (142) believe that in F_1 crosses the genotype controlling chlorophyll concentration is formed by equal contributions from male and female parents. A continuation of the classical studies in modern form is given by Buvat (32) in which there are illustrations of the transformation of chloroplasts of mesophyll cells of *Brimeura amethystina* into amyloplasts during the time of petiole development. This is not, however, true of all chloroplasts, since those cells which have lost their somatic powers appear to be transformed into embryonic cells which can differentiate into new roots.

Two articles are available on chlorophyll concentration in polyploids. Györffy (92) reports that in general the pigment content of polyploids is greater than that of diploids. Another distinction made is that the chlorophyll *a/b* ratio in diploids is usually greatest in the spring-summer period, while with tetraploids the greatest ratio occurs during the fall-winter period. In a more careful analysis, however, Pirschle (171) points out that, although tetraploids are richer than diploids per unit area, they actually contain somewhat less chlorophyll on a fresh-weight basis. The reason, of course, is that tetraploid leaves are usually thicker. The leaves of tetraploids are, however, generally larger, and per leaf tetraploids contain more chlorophyll. No difference was found in the normal pigment concentration ratios.

It is the opinion of the reviewer that one of the most fruitful approaches in plant physiology on the mechanism of tissue differentiation is that given by White (231) in which it is shown that a variety of perturbing forces may result in tissue differentiation, e.g., into leaves. It is, therefore, of interest to note that Loo (130) has been able to cultivate excised stem tips of dodder for five months through several transfers. Both flowers and lateral buds were produced without development of leaves or roots, although a large amount of chlorophyll was developed. The primary growth requirement appeared to be sugar, lyophylised whole cytoplasm not being as effective. It is, however, impossible to culture for an indefinite time leaves which cannot produce roots and thus become entire plants again. This is true regardless of the age of the leaves, so that one cannot regard the lack of success as primarily due to a natural limitation of growth via inhibition. Rather there must be some material formed by the root which sustains growth. This appears to be a most interesting field for study.

The relative amount of chlorophyll within leaves of a plant is not, however, a direct indication of the amount of photosynthesis that may occur, a fact long ago shown by Willstätter and Stoll (234). In a variety of circumstances it was pointed out that the amount of photosynthesis per unit of chlorophyll was often greater in leaves with less chlorophyll. This is, of course, readily explained on the basis that the photochemical reaction occurs only on the surface of the chloroplast and that the efficiency of energy transfer from within the chloroplast to its surface is not 100%.

Under these circumstances the relative efficiency of photosynthesis would increase with decreasing chlorophyll content down to the region of depopulation of the chloroplast surface. Shcheglova (199) has shown another technical variation in buckwheat leaves where the amount of photosynthesis in early summer is greater with leaves containing a small amount of chlorophyll, the reverse being true in late summer. The effect, explained by her on the basis of a temperature effect, is also just as readily explained as a light effect, if the light is not saturating in early summer but is in late. Roubaix and Lazer (185) find no relation between chlorophyll content in leaves and sugar in the beet. The compensation points of a variety of sun- and shade-loving plants have been given (186). Comparisons have been made over a three-year period on chlorophyll, carbohydrates and water content of bean leaves grown in light and in partial shade (109). The chlorophyll ratio (sun to shade) was 1.3 on a fresh-weight basis, but 0.99 on a dry-weight. The corresponding average CO_2 assimilations was 1.14 and 0.82. The respiration is greater in the sun leaves. Emerson, Green and Webb (52) have pointed out in a more quantitative fashion that the maximum amount of photosynthesis per flash of light is not directly related to the amount of chlorophyll so much as it is to internal factors, e.g., previous conditions of growth (of algae), color and intensity of illumination, and age of culture. Pickett and Kenworthy (170) believe that in apple leaves the amount of "initially" exposed surface is more important for photosynthesis than the absolute amount of chlorophyll.

The amount of chlorophyll at various times in the life history of a plant, or under varying conditions, have long been popular studies; they continue to be. Zaitseva (238), for one, notes the increase in chlorophyll content of wheat leaves from vernalized winter wheat up to the point where the last leaf no longer envelopes the spike, after which the chlorophyll content begins to decrease. The decrease was not apparent in non-vernalized wheat. In rice, barley and wheat, Nagasima (154) reports two maxima, one during most active growth and the other during the reproductive season. The latter maxima is not a certainty. Olsen (164) finds only a single long-period maximum (June to autumn) for beech leaves. As in Iuracec's work (*loc. cit.*) chlorophyll content per unit surface is higher, though it is equal on a dry-weight basis, for leaves exposed to sun as compared to those in shade.

PATHOLOGICAL ASPECTS

The characteristic pattern produced is the simplest means of recognizing chlorophyll destruction caused by tobacco mosaic virus and has induced an interest in the mechanism of chlorophyll degradation. Dufrenoy (46) relates this to enhanced oxidase activity during virus infection. The increase in enzymatic action is presumably due to increased substrate arising from the degradations of natural proteins, etc., due to the virus. Because of the autocatalytic nature of the infection this enhancement never diminishes, and the chlorophyll is thus destroyed through oxidase activity (see section on enzymes). Actually we have no knowledge of the mechanism of chlorophyll degradation beyond that of the chlorophyllides, due to chlorophyllase. We have no indication whether degradation proceeds via oxidation or reduction and have not the slightest hint as to the nature of the lenco compounds. In contrast to Dufrenoy, Gol'din (79) presents data which indicate little difference in chlorophyll content between sound and affected plants, the weight, if any, being in favor of the affected leaves. He believes that the rate of formation of the virus does not always parallel the delay in development of the chloroplasts. At variance with both opinions, McKinney (133) states that not only did virus-infected tobacco have less chlorophyll* (30% less) as well as a decreased chlorophyllase activity, but also had decreased oxidase activity. In cotton with the virus producing leaf curling, Kar-Murza (113) reports less chlorophyll in infected than in healthy leaves. It is obvious that in the same plants with the same virus, the same results should be obtained. It is of course conceivable, however, that the virus in different strains acts either more slowly or in a different fashion, so that chloroplasts are affected either at different rates or so indirectly that the chloroplasts, which are to a certain extent independent, may not seem to be affected at all.

In fungus diseases it is possible, as Müller (152) has pointed out, that an organism can in one instance act as a symbiont, as *Marasmius oreades* on *A. millefolium,* even though chlorophyll content is reduced, but as a parasite on others.

Needless to say, the use of certain sprays or control chemicals, e.g., naphthalene acetic acid or Pb-arsenate (217), which affect the normal development of cells, may well be expected to affect the amount of chlorophyll development. It is interesting to note, however, that in the former, although chlorophyll is diminished, ascorbic acid is not. Pickett and Birkeland (169) believe that some of the lack of cell development of Pb-arsenate-sprayed leaves may be due merely to unavailability of surface layers of the palisade cells for normal reactions, not to a specific chemical effect.

ENZYMES IN CHLOROPLASTS

Included in this section are several papers, not necessarily related, which have in common the biochemical approach as well as the common spatial environment of the enzymatics involved.

Of primary interest, especially in view of the demonstrated ability of the photochemical reduction of quinones by chloroplast grana (10, 226–228), is the relationship between the naphthoquinone, vitamin K, and chlorophyll. Dam, Glavind and Nielsen (40) have shown that the chloroplasts of cabbage have 60 times as much vitamin K as the cytoplasm. Furthermore the vitamin K concentration is proportional to the chlorophyll concentration which in turn is generally proportional in young seedlings to the amount of light. It would be interesting to know whether light itself is really required for vitamin K formation as it usually is for chlorophyll[4]. A similar relationship has been reported for ascorbic acid, although here too the direct relationship is still in doubt. Continuing in this problem is the work of Neubauer (156), in which this relationship is reiterated and carotenoids are also mentioned in the same respect. It is of course well known that carotenoids always accompany chlorophylls, though the reverse is not by any means true.

Another parallelism which exists is that of catalase–chlorophyll (45, 166). Nakamura (155) and Kashimoto (114), continuing previous suggestions of the former, found that most plant catalase is in the chloroplasts in higher plants, and that in the lower as well as the higher the chlorophyll–catalase relationship existed. Similarly in chlorotic and aged yellowed leaves both have decreased. It is, however, well known that many if not all porphyrin compounds have catalase-like powers. It is interesting to note, therefore, that catalase itself actually exists in these tissues (114) and

[4] One may thus speculate that if the process of photosynthesis is merely the reverse of respiration (33) no basis is afforded for unique syntheses, since such reversals may occur, though to a lesser extent, in respiration itself. Either the compound(s) or its intermediate(s) in photosynthesis is in some manner different from those in respiration, or other unique photochemical reactions occur apart from photosynthesis as it is usually understood.

that chlorophyll is not performing the function assigned to catalase. Krossing (121) has described a method whereby green leaves are divided into four portions: chloroplasts, cytoplasm, water-soluble portion, and cell wall material. A study of the distribution of various enzymes showed that the chloroplasts alone contained the chlorophyllase and amylase, and contained the predominant amount of catalase, while the sole substance identified confined to the water-soluble fraction was the peroxidase; the cytoplasm also had significant amounts of catalase, both it and the chloroplasts possessing nominal amounts of sucrose. Sisakyan and Kobyakova (206) find invertase, amylase and protease in plastids. Yin and Tung (236) have provided a histochemical demonstration of the localization of phosphorylase in the guard cells of epidermal tissue.

Pallares *et al.* (167) have discovered a most interesting relation in amphitrophic *Euglena viridis*. In a medium containing glucose, cellobiose and lactose, and in media to which various vitamins were added singly (A, D, E, K—synthetic—, B_1, B_2, B_6, C, H, P, PP, pantothenic acid and inositol) and then kept in the dark for two weeks all cultures except those containing vitamin H (biotin) (1/100 ml.) and vitamin C (ascorbic acid) (200 mg./100 ml.) lost their chlorophyll. Assuming that the effect is not due to that of minor element contamination (e.g., Fe, Cu), the effect and the organism could be used as a means of tracing in part the mechanism of chlorophyll development. Elliott (51) notes an effect of growth stimulation on *Euglena* in light but not in dark with phytohormone. In this regard the work of Gustafson (91) is pertinent. He reports that corn seedlings, devoid of chlorophyll either because of being grown in the dark or because of genetic constitution, had more growth hormone than normal similar plants. The relationship is thus still obscure unless this is merely an instance of photosensitized oxidations. A possible cytogenetic effect of saponins is indicated in the work of Balansard and Pellissier (14). Algae (Protococcae) grown in saponin (quillaja and polygala) solutions (10^{-4}–10^{-5}) formed more chlorophyll than the controls. Furthermore ivy with mottled leaves put out new leaves which were entirely green.

Arnon (4, 5) has shown that chloroplast grana are the seat of the polyphenol oxidases in green leaves (chard).

Von Euler (56, 57) has noted in a series of papers that addition of streptomycin to germinating seeds results in a lack of chlorophyll formation. The mechanism is believed to involve disruption of nuclear functioning, especially of nucleic acids.

Among the more interesting of the enzymes present in many plants is that which hydrolyzes phytol from chlorophyll, i.e., chlorophyllase. In a study of this enzyme (229), which is active at room temperature in such strange solvents as 80% ethanol and 40–70% aqueous acetone as well as in water at 75°, the authors point out the variability both with single plants and among different plants as well as its difficulty of extraction.

Noack (160), continuing his previous studies on chlorophyllase, believes that it functions primarily in the decomposition of chlorophyll, since once the water-soluble chlorophyllide is formed, it can be more or less readily oxidized by peroxide. The conditions for this oxidation are believed to occur in conjunction with iron as colloidal ferric hydroxide. Indeed it is shown that it is possible to oxidize chlorophyllide completely to carbon dioxide, ammonia and water, with hydrogen peroxide and ferric hydroxide. The optimal conditions in a leaf at which this destruction may occur are those just before the leaves turn yellow (*sic!*), when catalase activity is diminished.

Boichenko (25) found that chloroplasts possess a hydrogenase system which could be activated by methylene blue. Both oxygen and carbon dioxide could be reduced by molecular hydrogen (the latter goes to formate even in the absence of oxygen). The reactions are reversible, and gaseous hydrogen may be produced for an extensive period.

JOINT ACTIVITY OF PIGMENTS

By means of action spectra it has been known since Englemann's time that in red algae containing phycoerythrin, a water-soluble bilin protein, that pigment is capable of taking part in the light reaction in photosynthesis. This is of extreme interest, indicating either that it is not an absolute prerequisite that chlorophyll take part in photosynthesis, or that a coupled reaction of some type occurs between the pigment systems, one of which is in the cytoplasm and the other in the chloroplasts. Haxo and Blinks (23, 70*a*) have recently shown that in the red algae, which contain in their chloroplasts the water-soluble bilin-proteins, phycoerythrin and phycocyanin, the more phycocyanin the less operative is the chlorophyll. In the genus *Porphyra,* containing the most phycocyanin, the chlorophyll is essentially inactive. Although under abnormal conditions where the phycoerythrin is leached out by the sun, the chlorophyll is again operative.

This is, therefore, the first authenticated instance of a genus containing chlorophyll which is non-functional. The authors give no explanation for the inactivity of the chlorophyll; indeed, the assumption had heretofore been made that these water-soluble pigments operated through the chlorophyll by transferring energy to them. Mathematically formal analogies in comparing this energy transfer to the process of internal conversion within the atom emitting gamma rays have been proposed (3*a*).

This analogy, while possibly of interest in relation to the similar problem of sensitized carotenoid transfer, appears, therefore, irrelevant when applied to the bilins. These pigments are, of course, closely related to the porphyrins, and there is no reason why they should not be capable of participating in the photochemical reaction *per se.* It is even possible that in a competition for the limited hydrogen acceptor the bilins should be superior to the chlorophyll because of their water-solubility, although this will not of itself explain the poor yield of chlorophyll action and still less its inactivity. The fact that after leaching of phycocyanin the chlorophyll is again functional, and that in other genera with lesser amounts of phycocyanin the chlorophyll is at least partially active, implies an actual inhibition of chlorophyll activity by phycocanin, possibly by competition for the catalytic sites on the protein surface. Increasing evidence indicates that within the chloroplast itself, other pigments, e.g., the carotenoids, may take part in photosynthesis. This point of view has been maintained qualitatively for some time by Montfort (145, 147). Quantitatively this has been presumably demonstrated (49) in the diatom *Nitzschia closterium,* in which, at those wave lengths where absorption of carotenoids is considerable and of chlorophylls minimal, ascribing the entire photosynthetic yield to the chlorophylls would imply an efficiency greater than 100%. A similar result was obtained for the alga *Chroococcus* (53). The manner of transfer of energy from the excited carotenoid to chlorophyll has not been postulated. No addition compounds are formed between chlorophyll and carotenes, since absorption spectra of mixtures are strictly additive (12). However, in the highly concentrated states existing within the cell, very strong interactions are not only possible but probable. There remain at least two additional explanations for this apparent relationship, both of which have been anticipated to some extent (48). First it is not at all impossible that the extinction coefficients of the pigments within the leaf do not correspond with the extracted material, so that proper assignment of relative absorption of light has not been made.

This difference may arise from either of two sources: *a*) association with other substances or with itself, or *b*) orientation of the chlorophyll so that it is not randomly distributed with respect to the vector of incident light. In the work of Dutton *et al.* (*loc. cit.*) and Dutton and Manning (*loc. cit.*), a complete negation of the effect of carotenoids would involve an increase in the extinction coefficient at 5,000 Å of 2–3-fold. This does not appear likely, nor are the differences of vitro shifts of absorption spectra, compared to those of extracted material, likely to account for differences of such a magnitude. A second alternative, foreseen though not discussed by these workers and suggested for *Avena* seedlings by Frank (67), is the spatial separation of the pigments within the chloroplast. It was found that despite large increases of carotenoids with time (double in the period of 72–120 hours), the effectiveness of the incident (blue) light for chlorophyll formation was unimpaired. These results were regarded as showing not only that the carotenoids did not screen out the light by being in front of the protochlorophyll and were not in statistically equivalent positions, but that they were actually situated behind the protochlorophyll. It is not impossible that the reverse is true in *Nitzschia*, at least for the carotenoid involved. Indeed, that some carotenoids screen, and others do not, may explain why some appear to be active, and others not. The conception appears, however, to be probable only in the case of a monolayer. Indeed a similar explanation for the effect could be ascribed to an adjacent disposition of the carotenoids. Either theory would appear to be precluded in case of multilayers or multicellular tissue unless there is a precision of alignment within the chloroplasts and of the chloroplasts within cells, of which we are not yet aware. A simpler explanation is that the light absorbed by the carotenoids is transferable to the protoporphine for chlorophyll formation.

OPTICAL ACTIVITY

Use of the optical activity of chlorophyll derivatives, which once played an important, though misleading, role in the determination of chlorophyll structure (see, e.g., the review by Fischer, in Chem. Rev. 1938), has had revived interest, especially in the determination of the degradation products of the oxidation of chlorophyll derivatives. Furthermore reports have occasionally been made of the inactivity of compounds which were expected to be active, with reappearance of activity on further transformations. Pruckner, Ostreicher and Fischer (179) have shown that a number of these anomalies are due to the effect of the wave length of the light used to take the measurement (because of the pronounced change in dispersion near an absorption band). It is, of course, an unfortunate habit that has arisen, of reporting optical activities at only one wave length, inasmuch as these are of no more value than if absorption spectra were given at but a single wave length. The effects of concentration are also given, the results of which may indicate varying degrees of association. In using a red filter (690–720 mμ), for instance, the following values are listed for methyl chlorophyllide *a*:

Mg. / 100 cc. acetone	$(\alpha)^{20^\circ}$
10.65	660
5.37	940
2.68	740

The results with mesopheophorbide are even more anomalous.

REVERSIBLE BLEACHING

Porret and Rabinowitch (175) found that in oxygen-free solutions (methanol) chlorophyll is reversibly bleached with strong light (ca. 1% with 2000-watt carbon arc, with a red filter). The effect was increased markedly with formic acid. The intensity of the light was such that for the concentration of chlorophyll employed, each molecule absorbed a quantum of light once a second; the color returned in the dark in about ten seconds. Livingston (125, 126) repeated the experiments, using more purified solutions and weaker light. Although the regeneration time was found to be much increased as a result of the purification of solvent (to 100 seconds), the stationary bleaching was confirmed to be proportional to the square root of the light intensity. This was interpreted by Rabinowitch[5] as a bleaching effect, proportional to the light intensity, and as a regenerating effect, proportional to the square root of the regenerating bleached products. The assumptions of Porret and Rabinowitch in this regard were confirmed by direct determination of the regeneration time in Livingston's work. Rabinowitch (*loc. cit.*) lists four possible mechanisms for chlorophyll bleaching: tautomerization, dismutation, oxidation, and reduction. The first should result in a monomolecular back reaction, which is not the case; the second is excluded on the basis of concentration (ca. $10^{-5}\ M$), although this possibility is not entirely ruled out. McBrady and Livingston (132) propose a combination of tautomerism and dismutation:

$GH + \text{lite} \longrightarrow GH^*$ (absorption)
$GH^* \longrightarrow GH + \text{lite}$ (fluorescence)
$GH^* \longrightarrow HG$ (internal conversion accompanied by tautomerism)
$HG \longrightarrow GH$ (spontaneous reversion)
$HG + GH \longrightarrow GH_2 + G$ (dismutation)
$GH_2 + G \longrightarrow 2\ GH$ (regeneration)

The possibility of reversible oxidation is minimized by the probability of the oxidation of the reduced product by oxygen, which inhibits the reaction, leaving oxidized chlorophyll. The effect of oxygen could then be explained only by a catalytic acceleration of the reaction.

The possibility of a triplet state for the excited state of chlorophyll *b* has been suggested by Calvin and Dorough (35). Since chlorophyll *a* has not yet been shown to exhibit such phosphorescence the nature of the excited state is still in doubt. Were such a state to exist, then, as Livingston suggests, the inhibiting effect of oxygen would be due to its paramagnetism, the field effects of which would diminish the lifetime of the excited state of the chlorophyll. Reduction, however, is another possibility, since this would leave only oxidized solvent in the presence of oxygen. Rabinowitch considers the possibility of the reaction of chlorophyll with solvent: $\text{Chph} + \text{S} \rightleftharpoons \text{r Chph} + \text{oS}$, to be an important alternative to consider. Indeed one can use the colorless triphenyl carbinols in methanol as analogues (99). The action of light in these studies was to eject an electron to form a colored compound:

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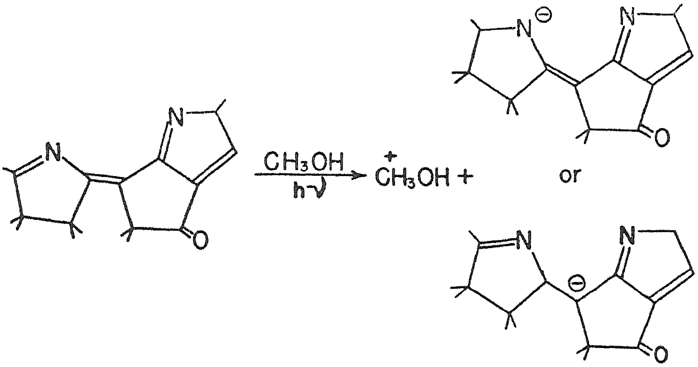
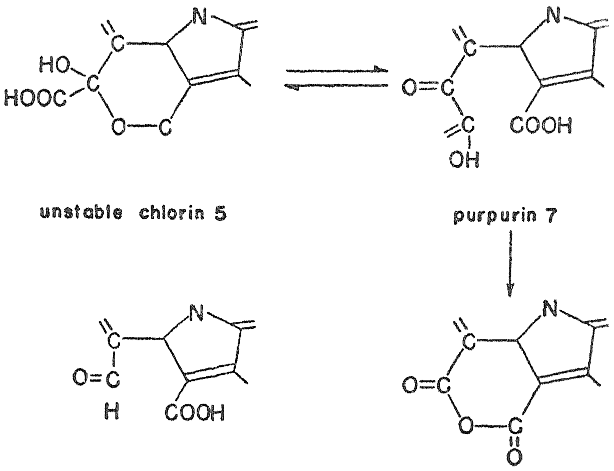
[5] For a thorough discussion up to the time of publication (1945), see Rabinowitch, "Photosynthesis", vol. 1, Interscience Publishers, N. Y.

$$CH_3OH + C_{31}H_{24}N_2 \longrightarrow C_{31}H_{25}N_2^+ + CH_3O^-.$$

The analogous reversed reaction could be conceived of as occurring at the δ-carbon or IV-nitrogen, resulting in loss of conjugation:



There is also the lesser possibility that the reaction may not be due to chlorophyll itself but to an equilibrium between some of its degradation products. More specifically, intermediates of the phase test, or allomerized material (both of which Rabinowitch indicates may be involved) give a direct example of the possibilities. Consider the phase test sequence: unstable chlorin 5 to purpurin 7 to purpurin 18; the corresponding absorption coefficients at the red maximum are $4.5 \cdot 10^4$, $3.0 \cdot 10^4$, and $5.8 \cdot 10^4$. If the action of light is involved in the equilibrium between the unstable chlorin and purpurin 7, then neither oxidation nor reduction is involved, though a large decrease in absorption coefficient occurs. It is interesting to note that the absorption coefficient of purpurin 5 at its maximum in the red is $4.5 \cdot 10^4$. The explanation of the reduction of the absorption coefficient by purpurin 7 would thus not be directly traceable to the alpha-keto group:



unstable chlorin 5

purpurin 7

purpurin 5

purpurin 18

In this regard the work of Calvin and Dorough (*loc. cit.*) is of interest. It was shown that a typical synthetic chlorin (Zn salt) could be photooxidized by quinone to the corresponding porphin. Here, too, traces of oxygen inhibit the reaction, and the explanation offered by Livingston for the oxygen effect is also employed here. Since it was found that the rate of the reaction was independent of quinone, down to very low concentrations, it was believed that the quinone did not compete with those mechanisms which deactivate the first electronic state (fluorescence, solvent, wall, etc.), but reacted with some other state, e.g., the triplet, obtainable from the excited state. The Zn chlorin was shown to be phosphorescent and possess a triplet-state lifetime of approximately 8×10^{-3} sec. Calculations showed that the time of active collisions between the chlorin and the quinone at the limiting concentration of the latter would be made in 10^{-5} sec. It was thus concluded that each triplet state molecule could react with the quinone until the concentration of the latter became so low that other deactivating processes occur. The Cu chlorin, which is not phosphorescent (or, if phosphorescent, of very short life-time) was also non-reactive photochemically.

Those experiments are of interest to chlorophyll studies, since there is no a priori reason why it should not act in a similar manner. Unfortunately chlorophyll *a* does not phosphoresce with a reasonably long life time, and probably some other type of longevity of an excited state, rather than the triplet, must be postulated [in this regard see the discussion by Franck and Livingston (66)]. It is of interest, however, that quinone can be used to effect the photodissociation of water, using chlorophyll grana, and under these circumstances the chlorophyll could well act as an H-carrier, e.g.,

$$\begin{array}{rcl}
\text{Chph} + h\nu & \longrightarrow & \text{H} + \text{``Photochph''} \\
\text{``Photochph''} + \text{H}_2\text{O} & \longrightarrow & \text{Chph} \cdot \text{O} \ (\text{or Chph} \cdot \text{OH}) \\
2\ \text{Chph} \cdot \text{O} & \longrightarrow & 2\ \text{Chph} + \text{O}_2 \\
\text{or } 2\ \text{Chph} \cdot \text{OH} & \longrightarrow & 2\ \text{Chph} + 2\ \text{OH} \cdot \\
2\ \text{OH} \cdot \longrightarrow \text{H}_2\text{O}_2 & \longrightarrow & \text{H}_2\text{O} + \tfrac{1}{2}\,\text{O}_2
\end{array}$$

Norris *et al.* (162) attempted by use of tritium to demonstrate the ability of chlorophyll to act as a hydrogen carrier, and were unsuccessful. Tritium is subject to difficulties both because of its large mass and because of attendant slow diffusion. Furthermore equilibrium constants involving tritium may be considerably less favorable than hydrogen. These authors suggested that use of 100% deutrium oxide could solve the problem unambiguously. Unfortunately this is not the case. Experiments with heavy water, and in conjunction with C^{14} (34), showed that, although there was more deuterium in the chlorophyll from algae exposed to light than in those kept in the dark, the amount of deuterium found was only ¼ of that expected. Furthermore the increased deuterium in the chlorophyll from the algae in light could be accounted for by increased synthesis (growth) in the light. These preliminary experiments indicate that chlorophyll may act as a sensitizer rather than as a hydrogen transport agent, or that, if it does act as a hydrogen carrier, *a*) it functions via an enolizable hydrogen, or *b*) the fraction of chlorophyll that is prominently active is small compared to the total amount, or *c*) the path of hydrogen from water to chlorophyll proceeds via a large reservoir of non-exchangeable hydrogen.

The reader is referred to Rabinowitch (*loc. cit.*) for an excellent discussion of earlier papers concerned with various photochemical studies on chlorophyll and significance of the apparently reversible bleaching of chlorophyll by Fe^{+3}. Of unique interest among these earlier studies is that by Gaffron (71) on the reversible photooxidation of isoamylamine with chlorophyll. In a typical experiment, in which 1 cc. of isoamylamine was added to an acetone solution containing 1 mg. of chlorophyll, with an atmosphere of oxygen, illumination of the system caused absorption of 288 cmm. of O_2 in $5'$. 95% of this oxygen could be released by addition of small amounts of MnO_2. Thus the chlorophyll sensitizes the photoformation of a moloxide. If water was present, H_2O_2 was formed. Addition of catalase to such a system would thus result in the continuous decomposition of water, analogous to the important type reactions involved in photochemical reduction.

PHOTOCHEMICAL REDUCTION BY CHLOROPLASTS AND GRANA

Englemann (54) showed, by means of bacteria motile only in the presence of oxygen, that the oxygen evolved during photosynthesis arose from the chloroplasts. That the gas thus evolved was oxygen was later shown by means of the color change of hemoglobin to oxyhemoglobin. Ewart (55) extended the earlier observation to isolated chloroplasts, in which, however, a greatly diminished activity, ceasing after a few hours, was noted. The experiments were in part duplicated and confirmed by Beyerinck (21), using luminescent bacteria which specifically require oxygen for luminescence, although only in small concentration, and the intensity of which may, to a certain extent, be used as a quantitative index of the amount of photosynthesis. More recently, using a phosphorescent dye quenched by oxygen, Franck (65) has measured the amount of oxygen evolved by chloroplasts quantitatively. The amounts of oxygen in these experiments were, however, of so minute an amount that ordinary Warburg manometric technique, in which one is concerned with ca. 10^{-6} mole of gas, was valueless.

It was thus of considerable interest that Hill (95) was able to show the evolution by chloroplasts, in light, of oxygen in the presence of ferric oxalate. The phenomenon was interpreted as a photolysis of water:

$$2\,Fe^{+3} + 2\,HOH \longrightarrow 2\,Fe^{+2} + 2\,H^{+} + O_2,$$

in which chlorophyll had served as a catalytic agent, and in which, because of the wave length of light used to effect the photodecomposition, energy had been transformed from electromagnetic to chemical, as in photosynthesis. Later, with Scarisbrick (98), the conditions of the reaction were extended so that, e.g., the rate of reoxidation of Fe^{+2} by oxygen, which limited the accumulation of the gas, was retarded by the use of ferricyanide. By the use of a variety of substances which normally poison or inhibit photosynthesis, azide, cyanide and fluoride, only the urethanes, which narcotize the reaction, were shown to have an inhibiting effect. Later work (e.g., 6, 103) conflicts on the inhibiting effect of azide. Although Aronoff obtained results in agreement with Hill, Arnon explains the lack of inhibition as due to the absence or low concentration of halide. This poses an interesting question as to why the reaction proceeds uninhibited by azide or hydroxylamine at low halide concentrations, albeit at lower values than if halide is present. The reaction was believed to be identical with that occurring during the light reaction in photosynthesis.

The sensitized photolysis of water is well known, e.g., that of ZnO or Ag_2O with UV, although, because of the wave lengths of the ultraviolet light used, energy is not stored. The reader is referred to the discussion of this and further examples, as mercury-sensitized decomposition of HOH, in Rabinowitch (*loc. cit.*).

Hill's work was extended in this country by that of French and co-workers (69, 70, 103–105, 123), who not only established the production of hydrogen ion in the reaction but further defined optimal conditions, such as pH, chlorophyll concentration and quantum yields, and gave proof of the source of the oxygen as being the water rather than the oxidant.

Further advances in this regard were made independently by Warburg and Lüttgens (226) and by Gurevitch (89, 90), who were able to show the photoreduction of organic compounds by chloroplasts in light, the latter having effected the reduction of dinitrobenzene to nitroaniline, and the former of quinone to hydroquinone. Warburg and Lüttgens, especially, showed the catalytic nature of the reaction, ten times as much oxygen having been evolved as there was chlorophyll present, and emphasized the energy storage of the process, as in photosynthesis. A later communication (227) showed that the reaction could also be obtained if the chloroplasts were broken down into smaller fragments, called grana, precipitable in nominal time by centrifugation only at relatively high speeds (ca. 20,000 g). Activity was shown to disappear by dialysis of the grana, and could be reinstated only on addition of minute amounts of halide (except fluoride) or nitrate ion. Arnon has recently shown (7) that the halide ion, not essential for plant growth, in some manner protects the photochemical system from the destructive effects of light. Furthermore the process was shown by Warburg and Lüttgens to be capable of poisoning with phenanthroline which was stated as being capable of removal and consequent reactivation of the reaction merely by washing. In their more extensive exposition (6) this was shown to be the remarkable effect of Zn which was more effective than excess equimolar amounts of iron. Arnon has also shown (6) that Cu is at least as effective as Zn, and that Co and Ni are more so. Both Warburg and, independently, Aronoff (10) showed that algae could evolve O_2 in the absence of external carbon dioxide. Fan, Stauffer and Umbreit (60) had previously shown that algae could reduce benzaldehyde and evolve oxygen thereby in the presence of light. Aronoff reported further the ability of other quinones, e.g., naphtha- and anthraquinones, to be used as oxidants. Correlations were made at different wave lengths with redox potential of the quinone-hydroquinone system, relationships existing in strong light being reversed in weak.

Gurevitch (*loc. cit.*), who demonstrated photoreduction by isolated chloroplasts of *o*-dinitrobenzene to *o*-nitrophenylhydroxylamine, showed that in the intact leaf the reduction proceeds to a further extent, namely to *o*-nitroaniline. The difference is presumed to indicate a loss of reduction potential on the part of the chloroplasts, due to their preparation, rather than loss of cytoplasmic factors which could effect such a reaction.

Boichenko (25, 26) has reported not only the oxidation of leuco dyes by chloroplasts but their assimilation of carbon dioxide, as evidenced by changes in the pH of the medium. This has not been confirmed (Aronoff, *loc. cit.*) and could not be shown [(29), Aronoff, 1947, unpublished] by means of $C^{14}O_2$. Any CO_2 uptake in Boichenko's experiments must thus be the result of a hydrogen-ion effect due to light (e.g., a photodecarboxylation) or a time change in the system. It is interesting to note that Warburg and Lüttgens' original contributions were entitled "Carbon Dioxide Assimilation", but no evidence was given of the inference.

Among the most important of the photochemical reductions is that in which an actual gain in free energy has been reported. Krasnovskii (120) reports photochemical reduction of riboflavin

($E_{pH=7} = -0.22$ volts) by ascorbic acid ($E_{pH=7} = +0.05$ v) in the presence of chlorophyll or Mg phthalocyanin. The reaction is reversible in the dark. There is no reason to believe that ascorbic acid, which is present almost solely in the chloroplasts and only so long as chlorophyll is present, does not perform a similar function in photosynthesis. In fact one may depict the beginning of a system of those substances known to be localized in the chloroplast and which may be involved in the photochemical reaction:

$$\text{R}^{*} + \text{ascorbic acid}^{\dagger} \xrightarrow[\text{light}]{\text{chlorophyll}} \text{RH}_2 + \text{dehydroascorbic}$$

$$[2\ \text{RH}_2 + \text{CO}_2 \longrightarrow (\text{CH}_2\text{O}) + \text{HOH} + 2\ \text{R}]$$

$$\text{dehydroascorbic} + \begin{array}{c}\text{polyphenols}\\ \text{or}\\ \text{naphthoquinols}\end{array} \longrightarrow \text{ascorbic} + \text{quinones}$$

$$\text{quinones} + \text{HOH} \xrightarrow[\text{light}]{\text{chlorophyll}} \text{phenols} + \text{O}_2$$

In any event this reaction will probably play an important role in elucidation of the photochemical mechanism in photosynthesis.

Occasionally reports have come of photochemical reductions involving synthetic states of chlorophyll [e.g., (17)]. A solution of chlorophyll in geraniol or phytol emulsified with glycerol containing some methylene blue was given an atmosphere of CO_2 and illuminated with a strong tungsten lamp, resulting in assimilation of some of the carbon dioxide and evolution of formaldehyde and oxygen. The present writer (1945, unpublished), repeating the experiments with ordinary Warburg technique, found only a strong photooxidation and utilization of oxygen instead of its evolution, but these experiments were of the static type, not of the flow methods used by the above authors. Zhukovskii (240) found that in using "strong" infra-red (presumably in the neighborhood of 3.5 μ, where CO_2 is a strong absorber), formaldehyde is produced by reaction of carbon dioxide with water. Inasmuch as this reaction requires a $\Delta F = + \sim 125$ kcal., this would require approximately 15 quanta, with light of the above-mentioned wave length, an extraordinarily inefficient reaction compared to known values for photosynthesis. Furthermore the photochemical reaction nominally implies an activation to an excited electronic state, in which higher frequencies would be involved instead of the vibrational-rotational level of this infra-red.

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* R is the cytoplasmic hydrogen acceptor (e. g., as Krasnovskii suggests, DPN or TPN).

† It is possible that a compound as aldopyruvic acid, which could be a photosynthetic intermediate, may be operative here also. Indeed, ascorbic acid may be formed in vivo from condensation of aldopyruvic acid and glyceraldehyde. The unique formation of ascorbic acid in the green plant tissues would thus be explained.

It is, however, established that a material may be prepared from leaves which does not contain any visible cell structure and which, though usable only as a colloidal suspension, appears to duplicate the photochemical reaction occurring in photosynthesis. It thus becomes possible to test various theories with regard to the function of chlorophyll in photosynthesis, with due regard to "foreign" substances still absorbed on the surface despite care of preparation, and to enzymes present but not taking part in the photochemical reaction. Examples of these questions which may be tested are *a*) whether chlorophyll acts as a hydrogen carrier or merely as a photosensitizer, *b*) whether phosphorus is involved in the photochemical reaction, and *c*) whether the chloroplasts are involved to any extent in carbon dioxide assimilation or merely serve as a source of reducing power.

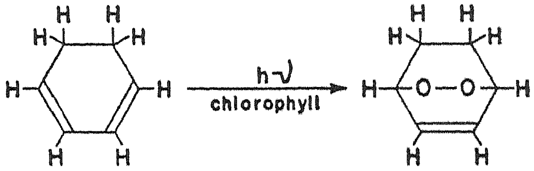
In other words the isolation of the cell fragments may serve as the first steps permitting elucidation of the biochemistry of the photochemical reaction in photosynthesis.

PHOTOOXIDATIONS

The ability of chlorophyll to act as a photosensitizer is known primarily because of its action as a photooxidizing agent. Examples of the purposeful use of chlorophyll in this manner are the following:

O. Richter (182) made use of chlorophyll solutions as sensitizers for gypsum photographic plates.

Schenk and Ziegler (187) have used chlorophyll solutions to effect peroxide formation in a number of analogous reactions of the type:



The quantum efficiencies of this reaction are quite high ($\gamma = 1$). Yamahuzi *et al.* (235) have demonstrated the photochemical production of H_2O_2 from aqueous acetone solutions in the presence of a sensitizer (chlorophyll) and a small amount of oxidizable organic matter (turpentine).

It is, of course, well known that chlorophyll itself is decomposed by light in the presence of oxygen, although it is quite stable in the absence of oxygen or solvents which do not contain peroxides. Aronoff and Mackinney (12) have studied the rates of photooxidation of purified chlorophylls *a* and *b* in benzene, using monochromatic light from a mercury arc (4358 Å). The reactions in both instances appeared to be of the second order and approximately equal, in contrast to the differing rates of pheophytin formation. The quantum efficiencies were very low, being of the order of 10^{-5}. Various substances, as carotene, afforded a certain amount of protection. In the presence of oxygen it could be shown that whereas some substances, as ascorbic acid, are photooxidized by chlorophyll, others are actually protected by the chlorophyll because of its absorption of near UV. Pepkowitz (168), using crude chlorophyll and non-monochromatic light, has conducted similar studies, primarily with respect to photochemical destruction of carotene.

Krasnovskii (119), previous to his classic experiment on photochemical reduction of riboflavin by ascorbate, showed that in the absence of oxygen, either ascorbic acid or phenylhydrazine causes loss of fluorescence and color, which can return in the dark. The chlorophyll, however, is altered, having lowered absorption coefficients both in the blue and, in the case of chlorophyll *b*, in the red.

Mention has previously been made of the sensitized photooxidation of allylthiourea (71).

Another sensitized photooxidation is that of phenylhydrazine by methyl red (73, 128). In this work it is shown that not all forms (ionic species) may take part, but only one, the zwitterion, or, as Livingston suggests, a non-ionic tautomer. This reaction, in which quantum yields up to 0.5 may be obtained, is independent of the chlorophyll concentration and, per unit of light, of the light intensity over a large range.

That chlorophyll photooxidation may be accelerated by ascorbic acid and inhibited by hydroquinone was shown by Aronoff and Mackinney (*loc. cit.*).

MECHANISM OF FORMATION OF CHLOROPHYLL AND RELATED COMPOUNDS

We are now reasonably certain that the immediate precursor of chlorophyll is protochlorophyll. We have almost no idea what the precursors, especially the colorless ones, of protochlorophyll are.

The speculations to be presented may not have real significance biochemically, but they have played a part in the repertoire of plant physiology. It is, therefore, not entirely wasteful to summarize, coordinate and evaluate these theories. There will, of course, come a time when our only direct means of solution, i.e., isotope and/or mutant investigations, will have to envisage these mechanisms in their larger aspect.

Theories concerning the mechanism of chlorophyll formation may be divided into three classes: *a*) speculations concerning the over-all method of formation up to a molecule as protochlorophyll; *b*) initial isotope experiments dealing with the utilization of metabolic intermediates, as glycine and acetate, in hemin synthesis, and which may not be directly applicable to chlorophyll; and *c*) physiological observations and correlations pertinent to chlorophyll formation.

Despite the wide-spread nature of the porphyrins, including chlorophyll, relatively little is known concerning the mechanism of their formation. It is known, for example, that iron is required, not only because of its intrinsic nature in the respiratory enzymes and hemoglobin, but also for chlorophyll formation in plants. Similarly Cu is necessary for both chlorophyll and hemoglobin formation. Until recent introductory isotopic experiments, nothing at all was known of the possible organic intermediates in its formation, for neither pyrrole[6] nor any of its substituted compounds from which porphyrins may be synthesized are found in nature. The question of the mechanism of porphyrin formation must also solve an additional problem, that of absence of some of the possible isomers (generally, 2 of 4) and predominance of one of those which do exist. More specifically it concerns the four isomeric types, corresponding to etioporphines I to IV, only types I and III of which occur in nature, and of these, type III predominates, except under some pathologic conditions. Theories which have been advanced regarding porphyrin formation will be considered first.

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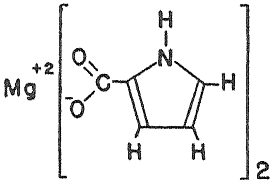
[6] Occasionally, as in the work by Troensgaard (223), one finds reports of pyrrole rings in proteins. Since it is well known that dry distillation of various substances, e. g., proteins, glutamic acid, etc., yield pyrroles (witness the process by which pyrroles are (were) manufactured by dry distillation of bones)—efforts must be made to prove their natural occurrence.

THE IRON-PYRROLE THEORY. A theory advanced (173) in 1915 enunciated the role of Fe in plants as that of producing pyrrole derivatives from non-cyclic compounds which follow some undetermined path to chlorophyll. Evidence for this consisted in the greening of Fe-chlorotic plants which had been fed Mg-pyrrole-2-carboxylate (IV):



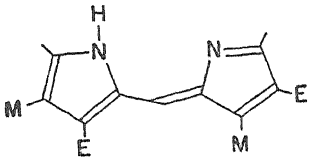
IV

Because of objections to this theory, two additional papers confirming the original work were published in 1935 (172, 174) and still another in 1938 by Lodoletti (129) who substituted 2-acetyl pyrrole for the carboxylic derivative with identical results and claimed an extension of the theory to any pyrrole derivative, such as acetyl-methyl-indole and acetyl-methyl-benzopyrrole, despite their insolubility in aqueous solution.

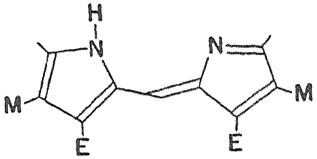
Deuber (43), using some pyrrole-2-COOH obtained from Oddo in addition to his own, not only was unable to obtain greening but found definite toxicity at lower levels than were specified for the original experiments. Similarly Demidenko (42) and Godnev (75) were unable to confirm the theory. The author (9) has found that neither pyrrole-2-aldehyde nor pyrrole-2-COOH can substitute for Fe in chlorophyll formation; nor can sodium bilirubinate, tryptophane, succinimide or mesoporphine serve in that capacity.

On feeding pyrrole-2-aldehyde to either *Chlorella* or barley roots (9), a purple pigment is developed. It is possible that this pigment is a porphyrin and that plants do have the capacity to produce porphyrins from pyrroles. It is, however, also certain that if the function of iron is to produce pyrroles, these latter are not in themselves sufficient to produce chlorophyll.

THE PYRROMETHENE THEORY. That porphyrins may arise from pyrromethenes has been postulated on two accounts, *a*) because of the terminal degradation of hemin in the animal to pyrromethenes, and *b*) because it offers an explanation of the dualism of the porphyrins in nature (i.e., types I and III[7] on the basis of etioporphine). Thus Dobriner (44) proposed two types of pyrromethenes (V and VI) which yield porphines of types I, II or III, depending upon whether they combine with themselves or with each other. However, neither etioporphine II nor its derivatives have been found in nature. The manner of origin or of the coupling of the pyrromethenes is not discussed.



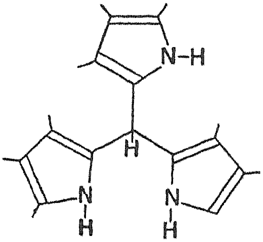
V



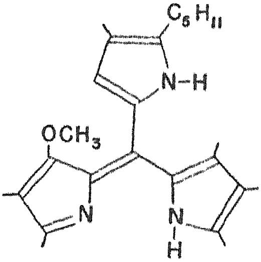
VI

M = methyl
E = ethyl

Turner (224) suggests a manner in which different pyrromethenes may arise from a single source, namely, a tripyrrylmethane (VII), akin to the pigment of the bacterium *Bacillus prodigiosus* (VIII). By rupture of any one of the three carbon-nitrogen bonds, three different pyrromethenes may be formed.



VII



VIII

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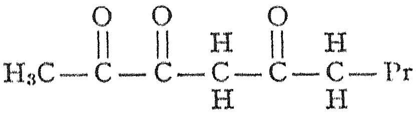
[7] The four etioporphines are:
Type I : 1,3,5,7-methyl, 2,4,6,8-ethyl porphine
" II : 1,4,5,8- " , 2,3,6,7- " "
" III: 1,3,5,8- " , 2,4,6,7- " "
" IV: 1,4,6,7- " , 2,3,5,8- " "

Turner further suggests that tryptophane may be the initial compound which, following a series of oxidations, may be transformed to a pyrrole.

A number of difficulties are involved in this theory, e.g., one of the indicated ruptures leads to a pyrromethene, condensation of which leads to type II etioporphines. Presumably one could say that in the organism this manner of rupture is enzymatically prevented. Secondly, although dipyrromethenes are well known in nature as breakdown products of bilins, tripyrromethenes are not. Indeed the bacterial pigment mentioned above is the sole example of its type known in living matter. Thirdly condensation of pyrroles to tripyrrylmethanes must also be viewed with some doubt inasmuch as, as previously mentioned, single pyrroles (not pyrrolidines) have not as yet been found in nature.

THE CARBOHYDRATE THEORY. Thomas (222) also considered the possibility of the oxidation of tryptophane to pyrroles in porphinogenesis. He showed quite definitely, however, that tryptophane was unable to generate protoporphine in the Harderian gland of the rat. [It did exert an apparent excitatory effect on hemoglobin formation, as noted by a number of others (1, 93, 138).] Nor did proline or oxyproline increase the glandular porphine. By utilization of various types of diets he reached the conclusion that porphyrin formation is for the most part effected by simple carbohydrates without specific characteristics, and not by particular substances. That is to say, the metabolism of carbohydrates provides building units for porphine synthesis, and these units are not as yet known.

It is a well-known fact that animal tissues are generally unable to synthesize aromatic units, as the benzene ring, thus resulting in, e.g., the indispensability of some of the amino acids. Thus a variety of theories have been proposed in which sugars and sugar derivatives have been made the basis of porphine synthesis. For example Robinson (183) has suggested the combination of a "hexose" and a "triose" (in an unspecified manner) to a high state of oxidation, resulting in the compound:

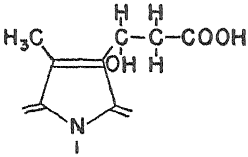


$$H_3C-\overset{\overset{\displaystyle O}{\|}}{C}-\overset{\overset{\displaystyle O}{\|}}{C}-\underset{H}{\overset{H}{C}}-\overset{\overset{\displaystyle O}{\|}}{C}-\underset{H}{\overset{H}{C}}-Pr$$

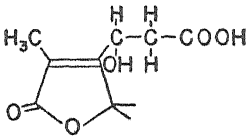
Four of these units are then combined, and, following addition of nitrogen via ammonia, dehydration and two isomerizations, a vinyl porphine may be obtained.

It is of course a simple matter to show on paper (8) that chlorophyll may be divided into four identical units (IX) whose propionic acid residues may be transformed by relatively simple biological reactions to the known residues of chlorophyll.

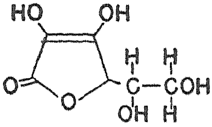
It is interesting to speculate that the initial condensing material is not of the pyrrole type, but of the lactone. This, then, suggests a moiety (X) similar to that of ascorbic acid (XI) from which pyrroles can be made readily via the well-known ammonium pyromucate synthesis of pyrrole itself.



IX



X

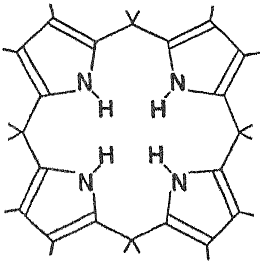


XI

Correlations between ascorbic acid content of leaves and the amount of chlorophyll at various stages and conditions have frequently been noted (59, 81, 143, 181, 225). This does not imply that ascorbic acid itself is directly involved in chlorophyll synthesis, although it may play a dual role. For example, Gortikova and Sapozhnikov (81) have shown the dependence of greening in plants upon the redox potential. By addition of ascorbic acid in optimum amounts at various pH levels to etiolated leaf blades in the dark, followed by exposure to light, a maximum amount of chlorophyll was formed at pH 8.2. Other substances, as 2,6-dichlorophenol indophenol, may substitute for ascorbic acid in regulating the redox level. Using a variety of redox indicators, the rH maximum for chlorophyll formation was found to lie between 17 and 22. One may observe that a lactone of the type of ascorbic acid may serve in any or all of three manners: *a*) in an indirect manner: dehydroascorbic acid + oxidizing enzyme ⇌ ascorbic acid + reduced enzyme, *b*) directly, by conversion of the lactone to a pyrrolone, *c*) coincidentally, i.e., as an index of the degree of oxidation of sugars (e.g., to diketones or to lactones) necessary for the formation of porphyrins.

THE CAROTENOID THEORY. Von Euler (58) has suggested that the conjugated carbons of the carotenoids provide the framework of the porphyrin nucleus. It is a fact that carotenoids always accompany chlorophyll, although the reverse is not true. While it is more probable that there is a relationship between phytol and the carotenoids than between the latter and chlorophyll, there is as yet no evidence that they bear any closer relationship than that of a common origin.

THE PORPHYRINOGEN THEORY. Godnev showed some time ago (76, 78) that it is possible to reduce protochlorophyll extracts with zinc and then reoxidize them in air to a green solution of identical absorption spectrum. He thus concluded that within the plant protochlorophyll arises from a colorless precursor of a character similar to the reduced protochlorophyll, probably a porphyrinogen (XII). It is a simple matter to form porphyrinogens chemically, although their oxidation to porphines proceeds only with difficulty and in poor yield. Smith (214) has shown, however, that there are ether-soluble, colorless, magnesium compounds in leaves, and it would be interesting to know, as a beginning, whether or not they also contain nitrogen.



XII

Indeed Granick (84, 85) has obtained orange-colored mutants (grown in light or dark) which contain protoporphine and Mg protoporphine, respectively, as their primary porphine pigments.

The interpretation of this is that in the production of the mutants from the wild type by UV, a gene in the sequence required for chlorophyll formation has been destroyed. Presumably this is the gene synthesizing the enzymes which convert in the first instance, protoporphine to Mg protoporphine; and in the second instance, Mg protoporphine to a precursor of protochlorophyll. Indeed, this precursor of protochlorophyll, Mg vinyl pheoporphine a_5 (protochlorophyll minus phytol), has recently also been found in very small quantities in another mutant (85*a*). A summary of the sequence of known compounds involved in chlorophyll formation is given by Granick as:

$$\text{glycine} + \text{acetate} \longrightarrow \text{protoporphine IX (heme minus Fe)} \xrightarrow{\text{Mg}}$$
$$\text{Mg vinyl pheoporphine } a_5 \xrightarrow{\text{4 or 5 steps}} \text{Mg protoporphine IX} \xrightarrow{\text{phytol}}$$
$$\text{protochlorophyll} \xrightarrow{2\text{H} \pm h\nu} \text{chlorophyll.}$$

While this type of reasoning is that normally employed in genetic studies of this kind, it appears also possible that a mutation has occurred which is esentially a rearrangement of a gene, rather than a complete elimination, so that the enzyme formed pursues a somewhat different pathway than that of the wild type enzyme. Under natural conditions for these organisms such a mutation would be lethal, but not under those used to induce growth. Thus while the above compounds may be intermediates in chlorophyll formation, the evidence is not yet conclusive. One must in fact reconstruct the entire chain.

The genetics of chlorophyll formation in corn indicate that there are at least twelve genes involved, and a variety of strains may be obtained with different levels of chlorophyll concentration. As an approach to the problem it is necessary to know whether or not the chlorophylls are identical in these different strains or whether a multiplicity of genes are required for maximum chlorophyll formation. If the latter is the case, then the actual biochemical steps from colorless precursor to protochlorophyll may be less than twelve.

It seems somewhat simpler, however, to approach the problem of chlorophyll formation by the use of organisms which form chlorophyll only in the light (e.g., *Euglena viridis*) or of mutants of wild types which normally form chlorophyll in the dark but now do so only in the light. Such organisms have recently been described by Davis (41) and by Granick (84, 85). It may well be that with these organisms the fortunate choice of a substrate whose formation was eliminated by mutation may again permit the formation of chlorophyll in the dark and thus provide an insight into the mechanism of chlorophyll formation.

Tuttle[8] has fed labeled (C^{14}) acetate to leaves and found that only the methyl carbon enters into chlorophyll formation. The extent to which the acetate molecule itself enters into the formation of the pigment is not known. As similar experiments (vide infra) with hemin indicate, the acetate may well be metabolized into other substrates used more directly. Recent papers dealing with the mechanism of hemin formation are listed in the bibliography (2, 24, 87, 150, 151, 200, 200*a*).

Shemin and Rittenberg utilized glycine containing N^{15}, and fed to rats the following compounds containing labelled N: glycine, ammonium citrate, *dl*-glutamic acid, *dl*-proline, and *dl*-leucine. The feeding was done over a three-day period, following which the normal rations were continued for two weeks before the hemin from the animals was analyzed and the results expressed as the ratio of $\frac{N^{15}\text{ conc. in hemin} \times (100)}{N^{15}\text{ conc. in compound fed}}$. The results for the respective compounds are 0.91, 0.09, 0.17, 0.18, 0.07. It was, therefore, apparent that glycine was the most effective source for the nitrogen of the porphine among the ones used. It did not prove, of course, that other amino acids, e.g., alanine, would not be equal or superior, or, indeed, that other types of nitrogenous compounds also were not effective. Furthermore the glycine may have served merely as an intermediate for rapid metabolism, e.g., transamination.

It has recently been shown (200*b*) that alanine will not substitute for glycine, nor will serine, alanine, aspartic acid or acetyl glycine. Although conversion of serine to glycine is known, the rate of incorporation of the glycine to porphyrin is so much more rapid than the conversion of serine to glycine that it is not possible to "dilute" the radio glycine by the addition of serine.

Blotch and Rittenberg, using deutrioacetate, showed that deuteriohemin was formed, with a D-content of hemin above that of the equilibrium D level of the medium. This indicated that the methyl carbon of the acetate, though not necessarily acetate itself, is involved in reactions which cause it to be synthesized into a methine carbon in the porphine ring, or a substituent on one or more of the pyrrole rings, since these positions contain the only hydrogenated carbons in hemin. Because of the known conversion of acetate to glycine, a reaction mechanism for synthesis of the pyrrole ring was given as

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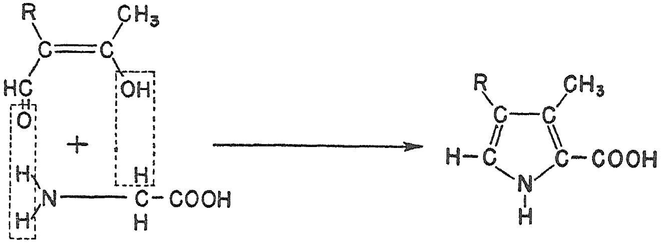
[8] Personal communication.



This mechanism is presumably intended to apply only to the chemical nature of the intermediates; otherwise the mechanism itself is open to the objection given above for individual pyrroles.

Grinstein *et al.,* in extending the above experiments, used carboxyl-labeled glycine and could not find evidence that it was used in hemin synthesis. Altman *et al.,* however, using methyl-labeled glycine, did find it to be preferentially used in hemin synthesis. Because there is no unequivocal evidence that the carboxyl-carbon of glycine (or acetate) is involved in hemin synthesis, it becomes necessary to *a*) label all three atoms involved, i.e., to synthesize, e.g., deuterio, carboxy C^{14}, N^{15}-glycine and determine whether all are incorporated in hemin in the same ratio, and *b*) to degrade hemin, e.g., as described in the porphine degradations of chlorophyll chemistry, and find where in the molecule the glycine or its parts are involved.

More recent work (87*a*) with C^{14}-labeled glycine has proved that the carboxyl carbon is not incorporated into the hemin, though it is into the protein, nor is there exchange of such carbon from the protein to the hemin. Thus it is evident, as these authors point out, that only the $-\overset{H}{\underset{H}{C}}-NH_2$ part of glycine is utilized for hemin formation. There still remains to be proved that this group enters intact.

Such evidence has recently been provided for hemin by Muir and Neuberger. These workers used not only labeled glycine, but also ethanolamine, which they believed might be one of the metabolic products derivable from glycine. The ethanolamine was incorporated only poorly into porphine. They established the conditions for the chromic acid oxidation of mesoporphine[9] on a semi-micro scale. In this isolation the N's of rings I and II are indistinguishable, as are those of III and IV. With the methylene-labeled glycine, the N's in the two groups of rings were found to be identical. On the basis of the atom % of the extracted hemin, taking into account its rate of formation, and the N^{15} content of the glycine pool, it was believed that the N^{15} must be incorporated into all the N's and therefore not only that $N_I + N_{II} = N_{III} + N_{IV}$ but that probably $N_I = N_{II} = N_{III} = N_{IV}$.

In an important abstract (151) Muir and Neuberger report results of the continued study of hemin formation, using doubly-labeled glycine ($N^{15}H_2—C^{14}H_2—COOH$) and labeled acetate ($CH_3—C^{14}OOH$ and $C^{14}H_3—COOH$). The oxidation products of mesoporphine, itself derived from hemin, are hematinic acid (rings III and IV), methylethylmaleic imide (rings I and II) and $BaCO_3$ (primarily methine carbons). With the labeled glycine, and assuming all the porphyrin nitrogen to arise from the glycine, it was found that

$$\frac{\frac{(C^{14})}{(N^{15})}\text{ porphyrin}}{\frac{(C^{14})}{(N^{15})}\text{ glycine}} = 2.4.$$

Further the specific activity of the imides was equal and about twice that of the $BaCO_3$. It is thus apparent that the methylene carbon of glycine can be incorporated into both ring- and methine-carbons of hemin. It is also apparent that glycine is further metabolized in some manner so as to lose some of its nitrogen prior to incorporation into the hemin. With the methyl-labeled acetate, incorporation of carbon into the rings is approximately equal for all rings, but does not enter at all into the methine carbons. Finally, with carboxyl-labeled acetate, although practically no incorporation into methine carbon occurs, utilization into the rings is unequal, the specific activity of rings III and IV being about four times as great as those of rings I and II. A comparison of the incorporation of carboxyl versus methyl-labeled actate (both hemins were initially of similar specific activities) for the two groups of rings shows roughly the indicated values of Table II. Since rings III and IV of hemin contain propionic acid residues, it is reasonable to conclude that the carboxyls of the acetate are transformed into the terminal carboxyls of the propionic acid.

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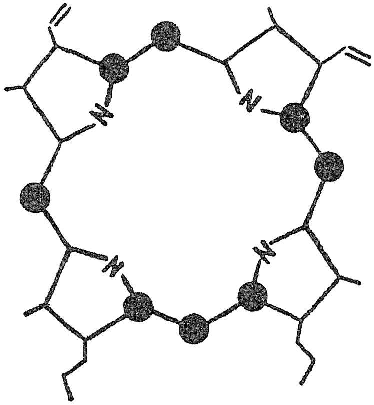
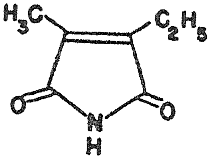
[9] [Mesoporphine IX = 1,3,5,8–tetramethyl, 2,4–diethyl, 6,7–propionic acid porphine. The mesoporphine is obtained by catalytic reduction of protoporphine; the latter is hemin minus Fe.]

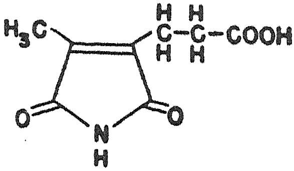
In a more recent abstract Wittenberg and Shemin (234*a*) state that α-carbon of glycine enters into the porphyrin molecule at the positions indicated in the diagram. The carbons of acetate are said to account for "most of the rest" of the positions. Details are not yet available.



XIII
heme skeleton



XIV
methyl ethyl maleic imide



XV
hematinic acid

Chlorophyll also contains a propionic residue on C_7 and an equivalent structure on C_6, i.e., the isocyclic ring. The failure of Tuttle to obtain incorporation of carboxyl-labeled acetate is therefore not clear. Again, reasoning from Table II, assuming the validity of a direct quantitative comparison, one would infer that the methyl carbons became the α and α' carbons of all the rings. It is pure speculation to assign positions to the carboxyl carbons, aside from the carboxyl of the propionic acid residue in rings III and IV. In rings I and II, however, one presumes they are associated with the vinyl groups.

Connected with formation of chlorophyll is the problem as to whether or not there is a daily variation, which has been reported by a number of workers, often with disregard for the wet weight (e.g., 30, 31, 189). Others (e.g., 37) imply a similar state when the notion of constant (usually implied as rapid) formation and destruction of chlorophyll exists. That rapid change does not exist can be shown by a calculation of the specific activity of the chlorophyll formed during photosynthesis of barley with radioactive carbon dioxide (11) which is, e.g., about 1/1000 that of the sugar formed. Later experiments (33) have shown that incorporation of both C^{14} and D into chlorophyll of *Chlorella* proceed in the ratio required by the empirical formula but less than that demanded by the composition of *Chlorella*. Net formation of chlorophyll thus appears to proceed faster in the dark than in light in these organisms.

TABLE II

	relative activity of	
	III & IV	I & II
carboxyl-labeled acetate	8	2
methyl-labeled acetate	4	4

Zaitseva (239) has shown that the removal of the endosperm from wheat seedlings diminished the amount of chlorophyll formed in light only if external sucrose was not supplied. A source of chemical energy is thus required. As mentioned earlier, Gortikova and Sapozhnikov (*loc. cit.*) have shown a dependence of chlorophyll formation, in both rate and amount, upon the redox potential which is determined by regulation of the pH and by ascorbic acid.

The claim is indeed made that the ascorbic acid state thus regulates chlorophyll formation. Oxygen, which is also known to be prerequisite for chlorophyll formation, could, however, be replaced by an organic hydrogen acceptor, as 2,6-dichlorophenol-indophenol.

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